Immunoneutralization of the aminoprocalcitonin peptide of procalcitonin protects rats from lethal endotoxaemia: neuroendocrine and systemic studies

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ABSTRACT

Severe sepsis and septic shock are an important cause of mortality and morbidity. These illnesses can be triggered by the bacterial endotoxin LPS (lipopolysaccharide) and pro-inflammatory cytokines, particularly TNF-α (tumour necrosis factor-α) and IL (interleukin)-1β. Severity and mortality of sepsis have also been associated with high concentrations of N-PCT (aminoprocalcitonin), a 57-amino-acid neuroendocrine peptide derived from ProCT (procalcitonin). Previous studies in a lethal model of porcine polymicrobial sepsis have revealed that immunoneutralization with IgG that is reactive to porcine N-PCT significantly improves short-term survival. To explore further the pathophysiological role of N-PCT in sepsis, we developed an antibody raised against a highly conserved amino acid sequence of human N-PCT (N-PCT-(44–57)). This sequence differs by only one amino acid from rat N-PCT. First, we demonstrated the specificity of this antibody in a well-proven model of anorexia induced in rats by central administration of human N-PCT-(1–57). Next we explored further the therapeutic potential of anti-N-PCT-(44–57) in a rat model of lethal endotoxaemia and determined how this immunoneutralization affected LPS-induced lethality and cytokine production. We show that this specific antibody inhibited the LPS-induced early release of TNF-α and IL-1β and increased survival, even if treatment began after the cytokine response had occurred. In addition, anti-N-PCT-(44–57) may increase long-term survival in LPS-treated rats by up-regulating the late production of counter-regulatory anti-inflammatory mediators such as ACTH (adrenocorticotropic hormone) and IL-10. In conclusion, these results support N-PCT as a pro-inflammatory factor in both the early and the late stages of lethal endotoxaemia, and suggest anti-N-PCT as a candidate for septic shock therapy.

INTRODUCTION

Septic shock is a systemic response to severe bacterial infections generally caused by the release of bacterial LPS (lipopolysaccharide) endotoxin, which triggers the release of pro-inflammatory cytokines, including TNF-α (tumour necrosis factor-α) and IL (interleukin)-1β [1]. Counter-regulatory anti-inflammatory mediators,

Key words: adrenocorticotropic hormone (ACTH), aminoprocalcitonin peptide (N-PCT), cytokine, mortality, procalcitonin (ProCT), septic shock.

Abbreviations: aCSF, artificial cerebrospinal fluid; ACTH, adrenocorticotropic hormone; CT, calcitonin; CTECCP-1, CT:carboxypeptide-1; CV, coefficient of variation; i.e.v., intracerebroventricular(ly); i.p., intraperitoneal(ly); IL, interleukin; LD100, absolute lethal dose; LPS, lipopolysaccharide; N-PCT, aminoprocalcitonin; NRA, normal IgG rabbit antibody; PPCT, pre-procalcitonin; ProCT, procalcitonin; RT-PCR, real-time PCR; s.c., subcutaneous(ly); TNF-α, tumour necrosis factor-α.

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such as glucocorticoids and IL-10, normally suppress pro-inflammatory cytokine production to prevent excessive inflammatory responses [2]. These innate immune responses are critical for protection against lethal infection and tissue injury, but the progressive and uncontrolled production of inflammatory mediators may result in the systemic inflammatory response syndrome, severe tissue damage and septic shock [3]. Experimental models of septic shock have demonstrated that a single injection of LPS into animals can produce changes that are characteristic of septic shock syndrome, and lethal endotoxaemia has been extensively used as an experimental model of septic shock [4]. Significant advances have been made in understanding the role of pro-inflammatory mediators in the pathogenesis of sepsis, but effective therapies have not yet entered clinical practice [5]. A major difficulty in developing therapeutics is that pro-inflammatory cytokines (e.g. TNF-α and IL-1β) is that they are released early in the development of a systemic inflammatory response. This leaves a narrow therapeutic window for administration of inhibitors of TNF-α and IL-1β, which are not effective when delivered after the acute cytokine response has occurred [6].

Severity and mortality of sepsis have also been associated with high concentrations of members of the CT (calcitonin) peptide family derived from the Calc-1 gene, including ProCT (procalcitonin) and N-PCT (aminoprolactin) [7]. N-PCT was first described in rats as a 57-amino-acid neuroendocrine peptide derived from the N-terminal half of ProCT [8]. Synthesis of N-PCT is complex and starts with the translation of a 141-amino-acid pre-prohormone (PPCT (pre-procalcitonin)) and formation of its immediate precursor ProCT, a 116-amino-acid peptide [9,10]. ProCT undergoes enzymatic cleavage and it breaks down into multiple fragments. These include N-PCT and the conjoined CT:CCP-1 (CT:carboxypeptide-1)). Unlike CT:CCP-1, the N-PCT region has been highly conserved during evolution and its amino acid sequence is quite similar in both humans and rodents [11]. Expression of this group of peptides is normally limited to thyroid C-cells and, to a small extent, other neuroendocrine cells. Thus, in the absence of infection, the extrathyroid transcription of the Calc-1 gene is suppressed and all of these peptides circulate in low concentrations as free peptides in the serum of healthy individuals [12]. N-PCT has been identified as the most abundant CT/CT-gene-related peptide gene product in normal serum [12,13].

During severe systemic inflammation, in particular related to bacterial infection, the tissue-specific control of Calc-1 breaks down and there is an increase in Calc-1 gene expression which causes a release of ProCT and N-PCT from all parenchyma tissues and differentiated cells types throughout the body, without corresponding CT secretion [14], and, more importantly, their levels persist for relatively long periods of time and correlate with sepsis severity and mortality [15,16]. Thus it has been suggested that ProCT in sepsis and systemic inflammation is a harmful biomarker and a therapeutic target in humans with sepsis [17]. However, researchers have not determined the exact role that ProCT plays in the pathogenesis of sepsis, and a number of unanswered questions that merit further pathophysiological studies remain regarding the biological role of CT precursors, including ProCT and N-PCT, during sepsis.

In sepsis, the extent to which any specific CT precursor peptide is increased relative to the other varies [12,18]; indeed, the levels of N-PCT and CT:CCP-1 may be even higher than the ProCT values [12,18]. The inflammatory release of N-PCT can be induced in two main ways: one is due to the release of LPS, and the other one is through a cell-mediated host response stimulated by pro-inflammatory cytokines (in particular TNF-α) [11,19]. Furthermore, it has been shown that N-PCT has leucocyte chemoattractant properties and modulates LPS-induced CD11b up-regulation in activated monocytes and neutrophils [20]. Previous studies in a porcine model of lethal polymicrobial sepsis have revealed that both the early and late immunoneutralization with a purified IgG that is reactive to porcine N-PCT (amino acids 32–45) improved the physiological and metabolic parameters of pigs with sepsis and greatly increased their short-term survival [21,22]. Previous in vivo studies in rats indicate that N-PCT is expressed in specific brain regions and that central administration of N-PCT to rats recapitulates many of the clinical signs of sepsis, including lethargy, fever, anorexia and body weight loss [23]. These sickness-type behaviours were mediated via prostaglandin-dependent inflammatory pathways and correlated with a marked activation of hypothalamic nuclei of primary importance in regulating energy homeostasis during sepsis, where LPS and pro-inflammatory cytokines (e.g. IL-1β, IL-6 and TNF-α) mediate effects on energy balance [24]. Taken together, these findings suggest that N-PCT plays a relevant role in the body’s inflammatory response to sepsis.

To explore further the pathophysiological role of N-PCT in sepsis, we developed an antibody raised against a highly conserved terminal fragment of N-PCT (amino acids 44–57) in both humans and rats. In the present study, we tested the effects of this antibody in a model of lethal endotoxaemia and determined how this immunoneutralization affected LPS-induced lethality and circulating levels of pro- and anti-inflammatory mediators. We report that both prophylactic and therapeutic treatment of endotoxaemic rats with this specific antibody against N-PCT [anti-N-PCT-(44–57)] attenuated morbidity and markedly increased their survival by down-regulating the innate inflammatory response.
MATERIALS AND METHODS

Reagents
The polyclonal rabbit anti-(human CT precursor) used in our studies was synthesized by AbD Serotec (1720-9670). The immunogen was used as a synthetic peptide corresponding to amino acids 69–82 (E69QEREGLDSRSPS57) of human PCT, the native molecule of N-PCT. According to the BLAST protein database search program, this sequence corresponds to a specific and highly conserved N-terminal segment of human N-PCT (amino acids 44–57) and differs from that of the rat sequence by only one amino acid (E44QEAEGGLDSRPS57). Furthermore, it does not have alignments with any other member of the CT/CT gene-related peptide family.

For central studies, purified human N-PCT (A1PFRSALESSPADPATLSEDEARLLLAAALVQDYVQMKA-SELEQEQEREGLDSRPS57; Bachem) was used because previous studies have used this form of N-PCT [23,24], therefore enabling us to compare our results to other published work. Reverse-phase HPLC, amino acid sequencing and MS ascertained the purity of the peptide. N-PCT and anti-N-PCT-(44–57) were dissolved in a microfiltered and sterile solution of rat aCSF (artificial cerebrospinal fluid; Harvard Apparatus). Heat-inactivated N-PCT-(44–57) (90 °C for 1 h) and NRA (normal IgG rabbit antibody; Sigma–Aldrich) were used as controls.

For peripheral immunization studies, lethal endotoxaemia was induced by bacterial LPS (Escherichia coli serotype 0111:B4; Sigma–Aldrich) was dissolved in PBS and administered i.p. (intraperitoneally). PBS and NRA were used as controls. Furthermore, in some experimental groups, we administered i.p. a mouse monoclonal anti-ProCT antibody (6f10; Abcam) to determine how this immunoneutralization affected LPS-induced lethality in comparison with a similar dose of anti-N-PCT-(44–57).

In these experiments, the immunogen used was a synthetic peptide corresponding to the entire molecule of human N-PCT (amino acids 1–57), and an isotype goat anti-(mouse IgG1) antibody (Biogenex) were used as a control.

All solutions were passed through 0.22 μm-pore-size Millipore filters and stored at −80 °C. The same batches and solutions of N-PCT and antibodies were used for all experiments and contained an endotoxin level <0.1 ng/ml of protein (i.e. below the detection limit of endotoxin assay), as determined using the Limulus lysate assay (ICN Biomedicals). Fresh aliquots were thawed before each experiment, briefly sonicated and warmed to 37 °C just before use.

Animals and housing
Adult male Wistar rats (Center for Animal Production and Research, University of Seville, Seville, Spain) weighing 225–250 g were maintained in individual cages under controlled temperature and light (lights on at 07:00 hours; lights off at 19:00 hours) with ad libitum access to tap water and a nutritionally balanced rodent diet (2014 S; Harlan Iberica). All experiments were performed at this ambient temperature of 26–28 °C (50 ± 5 % humidity), rather than at 20–22 °C, an ambient temperature below the zone of thermoneutrality for rats, and that could induce abnormal thermoregulatory responses and quantitatively accounts for most of the metabolic efficiency [25]. Furthermore, the administration of LPS was carried at the same time of day because of circadian rhythm effects on the inflammatory response [26]. Rats were randomly assigned to experimental conditions, and each rat was used only for a single experiment.

All experiments were done in accordance with guidelines on animal care and use established by the Council of the European Communities’ Directive (86/609/EEC) and Spanish regulations (BOE/67:8509/1988), and were approved by the Institutional Research and Ethics Committees of the Valme University Hospital.

Experimental design

Experiment 1: in vivo functional specificity of anti-N-PCT-(44–57) by determining the effects of i.c.v (intracerebroventricular) anti-N-PCT-(44–57) on N-PCT-induced anorexia

Before investigating the therapeutic potential of anti-N-PCT-(44–57) on LPS-induced overproduction of cytokines and lethality, the specificity of this antibody fragment was demonstrated by direct immunoneutralization of the central bioactivity of N-PCT on food intake in rats [23]. Chronic i.c.v. infusion cannulas and injections were performed essentially following standard surgical procedures [23]. Rats were anaesthetized with a ketamine cocktail (100 mg/ml ketamine; 5 mg/ml xylazine and 10 mg/ml acepromazine) and were placed in a stereotaxic frame. A stainless steel 26-gauge cannula was inserted into the lateral ventricle (1.1 mm lateral to bregma, 1.1 mm posterior to bregma and 3.5 mm ventral to dura mater). The cannula placement was secured with skull screws and cranioplastic cement. For presumptive analgesia, meloxicam [1 mg/kg of body weight, s.c. (subcutaneously); Metacam®; Boehringer Ingelheim], a non-steroidal anti-inflammatory drug used for post-operative pain in rats [27], was administered immediately after surgery while the rats were still under anaesthesia and recovering on a temperature-regulated heating pad. After surgery and recovery, each animal was housed singly throughout the experiments. Animals were acclimatized to the facility and handled daily for 1 week with unrestricted access to water and food prior to the onset of the experiment.

On the test day, groups of weight-matched (± 5 %) rats (n = 8 for each treatment combination) were infused i.c.v.
Experiment 2: murine model of lethal endotoxaemia and anti-(N-PCT) treatment

The purpose of these experiments was to establish a dose–response after LPS. Thus, before the induction of endotoxaemia, each animal was anaesthetized as above, and a temperature-sensitive radio transmitter (model PDT-4000; Mini-Mitter) was implanted into the peritoneum. Core body temperature (± 0.1 °C) was continuously monitored using the VitalView system (Mini-Mitter), as described previously [24,28]. Meloxicam (1 mg/kg of body weight, s.c.) was administered for post-operative pain control. After recovery, animals were acclimatized to the facility for at least 1 week with unrestricted access to water and food prior to the onset of the experiment (day 0). On day 0, the study animals were weighed and randomly distributed into groups of equal mean body weight (± 5 %). Rats were challenged in groups of ten with LPS (dose range: 1–15 mg/kg of body weight) by i.p. injection. Animals were observed for mortality for 7 days and feeding, movement and activity, and other clinical signs including ruffled fur, lethargy, appearance of diarrhoea and body weight loss. Any animal that appeared moribund or unresponsive to external stimuli before cervical dislocation, into tubes containing EDTA and cooled immediately at 4 °C. The plasma was separated by centrifugation (6000 g at 4 °C for 15 min) within the subsequent 15 min, and then frozen and stored at – 80 °C until assayed for cytokine and hormone concentrations.

Blood was collected, by cardiac puncture immediately before cervical dislocation, into tubes containing EDTA and cooled immediately at 4 °C. The plasma was separated by centrifugation (6000 g at 4 °C for 15 min) within the subsequent 15 min, and then frozen and stored at – 80 °C until assayed for cytokine and hormone concentrations. During necropsy, and at the same times after LPS injection, the brain was removed after retraction of the calvarium, placed with the dorsal aspects upward, and the hypothalamus was immediately dissected with a scalpel. The fresh hypothalamic block (average mass, 40 mg) was obtained with transverse cuts rostral to the optic chiasm and caudal to the mamillary bodies. Lateral cuts were made at the lateral sulci. The entire hypothalamus was immediately frozen in liquid nitrogen at the time of necropsy to preserve RNA integrity, and then stored at – 80 °C until protein extraction and
determination of hypothalamic Calc-1 expression and ProCT production by RT-PCR (real-time PCR) or by immunoluminometry respectively. We also studied plasma production of inflammatory mediators in long-term survivors pre-treated with anti-N-PCT-(44–57). Because immunoneutralization of N-PCT prevents LPS-induced mortality by approx. 85 % (see the Results section), a separate group of rats was used (n = 12). Survivors rats were killed at 3 and 7 days after injection (n = 4 per time point). Blood samples were collected and treated as described above for determination of cytokines and hormones.

Expression of the Calc-1 gene in hypothalamic samples
It has been shown that the Calc-1 gene is responsible for the synthesis of ProCT, and Calc-1 gene expression plays a role in the regulation of the neuroimmune response [14]. Thus to define the effects of anti-N-PCT-(44–57) on hypothalamic Calc-1 gene expression and ProCT production in vivo, we collected hypothalamic samples from rats at different time points after LPS injection in normal and immunized rats with anti-N-PCT-(44–57). Calc-1 gene expression was determined by RT-PCR at 1, 3, 6, 12 and 24 h after LPS (n = 6–8 rats per time point). A brain block containing the hypothalamus was rapidly dissected on solid CO2 and immediately frozen by immersion in liquid nitrogen. The frozen tissue was ground with a pestle and mortar under nitrogen. Total RNA from rat hypothalamus was extracted using the RNA from rat hypothalamus was extracted using the Roche Applied Science, and was normalized by the level of \\n
ProCT-like immunoreactivity in plasma and hypothalamus
Plasma and hypothalamic samples were assayed for ProCT using an immunocheniluminescence two-monomonclonal antiserum sandwich assay (BRAHMS Diagnostica), as described previously in hamsters [33] and rats [28] with sepsis. Hypothalamic samples were placed in 1M HCl, homogenized and centrifuged (10000 g for 30 min at 4 °C). Recovery after extraction was measured in hypothalamic samples at 1, 3, 6, 12 and 24 h after LPS (n = 6–8 rats per time point), and data were corrected for tissue protein concentration (Lowry assay; BioRad Laboratories). Hypothalamic supernatant and blood plasma were analysed in duplicate and repeated freeze–thaw cycles for reagents and samples were avoided. The assay for blood ProCT determination was adjusted to rat blood and is equivalent to human blood [CV (coefficient of variation) <15%]. This assay has a detection limit of 0.1 ng/ml. The intra- and inter-assay CVs were below 10%.

Measurement of cytokines, ACTH and CT in plasma
Determination of cytokine and CT levels in plasma was performed using commercially available rat ELISA kits for the detection of TNF-α (Pierce Biotechnology), IL-1β (R&D Systems), IL-10 (IBL), ACTH (Phoenix Pharmaceuticals) and CT (Phoenix Pharmaceuticals). Plasma cytokine levels in rats were measured 1, 3, 6, 12 and 24 h after LPS (n = 6–8 rats per time point), as described previously [28]. The detection limits were 15 pg/ml for TNF-α, 5 pg/ml for IL-1β, 10 pg/ml for IL-10 and 0.36 ng/ml for CT. The intra- and inter-assay CVs were below 5%. Plasma ACTH was extracted using a 1% trifluoroacetic acid buffer and C18 column technique. Extracted samples were dehydrated using an Eppendorf Vacufuge, and pelleted samples were stored at −80 °C. Samples were reconstituted 24 h before the enzyme immunoassay in assay buffer. The range of detection for the ACTH kit was 0–25 ng. All plasma samples were analysed in duplicate and repeated freeze–thaw cycles for reagents and samples were avoided.

Statistical analysis
Values are expressed as means ± S.E.M. Statistical analysis was carried out by ANOVA, followed by the Student–Newman–Keuls test in the case of pairwise comparisons between groups. Relationships were tested using the Spearman rank order correlation analysis. Survival data are presented as a percentage. Differences in survival were
determined by Kaplan–Meier analysis. Differences were considered significant when \( P < 0.05 \).

**RESULTS**

**Reversal of the effects of N-PCT on food intake by i.c.v administration of anti-N-PCT-(44–57)**

In order to assess the specificity of anti-N-PCT-(44–57), animals were injected i.c.v. with N-PCT in the presence or absence of anti-N-PCT-(44–57). Consistent with previous findings in rats [23], i.c.v co-administration of N-PCT [1 nmol/rat; 5 μg] and aCSF resulted in a reduction in the amount of food consumed by the animals, which was significantly different at 2, 4 and 24 h after the injection when compared with the counterpart aCSF-treated controls (ANOVA \( F_{3,31} = 10.9; P < 0.001; \) aCSF + N-PCT compared with aCSF + aCSF using a Student–Newman–Keuls post-hoc test, \( P < 0.001 \) for each; Figure 1A). Central administration of aCSF + heat-inactivated N-PCT did not cause any significant changes in food intake at 2, 4 and 24 h compared with aCSF + aCSF-treated rats (Student–Newman–Keuls post-hoc test, \( P > 0.05 \) for each).

When rats received anti-N-PCT-(44–57) (0.5, 1 and 2 μg/rat, i.c.v.) 30 min before i.c.v. N-PCT injection, the N-PCT-induced inhibition of food intake was time- and dose-dependently suppressed after treatment (ANOVA \( F_{4,39} = 114.2, P < 0.001 \)) (Figure 1B). Administration of 2 μg of anti-N-PCT-(44–57) reversed the effects of N-PCT on food intake, so that food intake was similar to the levels in aCSF-treated animals at 2, 4 and 24 h (Student–Newman–Keuls, \( P > 0.05 \) each). Thus, in the presence of this dose of anti-N-PCT-(44–57), the food consumed by the N-PCT-treated group was significantly increased at 2, 4, and 24 h (anti-N-PCT + N-PCT compared with NRA + N-PCT using a Student–Newman–Keuls post-hoc test, \( P < 0.001 \) each; Figure 1B). In a different set of animals, a lower dose of anti-N-PCT-(44–57) (1 μg, i.c.v.) was injected before N-PCT. In the presence of this dose of anti-N-PCT-(44–57), the food consumed by the N-PCT-treated group was also increased at 2, 4 and 24 h (anti-N-PCT + N-PCT compared with NRA + N-PCT using the Student–Newman–Keuls post-hoc test, \( P < 0.001 \) at 2 and 4 h, and \( P < 0.05 \) at 24 h; Figure 1B). A different group of animals was pre-treated with a lowest dose of anti-N-PCT-(44–57) (0.5 μg, i.c.v.). This dose of anti-N-PCT-(44–57) had no significant effects on N-PCT-induced suppression of feeding at 2, 4 and 24 h (anti-N-PCT + N-PCT compared with NRA + N-PCT using a Student–Newman–Keuls post-hoc test, \( P = 0.4, P = 0.1 \) and \( P > 0.3 \) respectively; Figure 1B). Treatment of rats with anti-N-PCT + aCSF or NRA + aCSF did not affect food intake when compared with aCSF-treated rats (Figure 1).

![Figure 1](image-url) Suppressive effect of anti-N-PCT-(44–57) on N-PCT-induced anorexia

(A and B) Food intake was measured at 2, 4 and 24 h after a single i.c.v. injection either 1 nmol (5 μg/rat) N-PCT or control [aCSF + heat-denaturized N-PCT and NRA + anti-N-PCT-(44–57)] or anti-N-PCT-(44–57) (0.5, 1 or 2 μg/rat) 30 min before i.c.v. administration of 1 nmol N-PCT in adult male Wistar rats. Because there was no significant difference between control NRA and anti-N-PCT-(44–57), these results were integrated into the same bar. Results are expressed as means ± S.E.M. (\( n = 8 \) animals per group). **P < 0.001 compared with N-PCT-treated animals (ANOVA, followed by a Student–Newman–Keuls post-hoc test).

**LPS induced morbidity and mortality in rats in a dose-dependent manner**

Figure 2 shows the dose–response curves for the mortality induced by LPS in male Wistar rats. We injected the animals with different doses of LPS (1–15 mg/kg of body weight, i.p.) and recorded survival rates. As shown in this Figure, rats receiving 15 mg of LPS/kg of body weight all died within 48 h, but in the groups that were given 1, 5 and 10 mg of LPS/kg of body weight the survival rates were 100, 80 and 40 % respectively. The Kaplan–Meier survival curve revealed significant differences in the median values among the treatment groups (\( P < 0.01 \)). On the basis of this experiment, we chose an LPS dose of 15 mg/kg of body weight (LD100) for drug screening in the next experiments. This dose of LPS induces profound inflammatory and immunostimulatory responses both in the periphery...
Aminoproc Alanction and septic shock

Figure 2  Survival rates of rats challenged with LPS
Rats were given LPS at doses of 1, 5, 10 or 15 mg/kg of body weight by i.p. administration. The results are expressed as the cumulative percentage of rats still alive within each interval, and the survival at 8, 12, 18, 24, 48, 72 and 168 h was recorded. Rat mortality was monitored twice a day for at least 7 days. There were ten animals in each group. The survival rate was estimated using the Kaplan–Meier method and compared using the long-rank test. **P < 0.01 and ***P < 0.001 compared with untreated rats.

and brain similar to pathophysiological changes found in human sepsis and septic shock [26,34]. All rats appeared less active and huddled together and showed symptoms of a septic-shock-like state, including lethargy, piloerection, chromodacryorrhoea and diarrhoea. As demonstrated previously by others [25], most of the animals exhibited profound hypothermia followed by fever at an ambient temperature within the zone of thermoneutrality for rats (see the Material and methods section). The animals were essentially moribund by 8 h after LPS and had a progressive mortality, reaching 20 % at 12 h, 80 % at 24 h and 100 % at 48 h (Figure 2).

Treatment with anti-N-PCT-(44–57) protects against LPS-induced toxicity
Because anti-N-PCT-(44–57) inhibits the N-PCT-induced effects, we expected a protective effect in high-dose endotoxaemia, a murine model for septic shock syndrome. Rats were treated i.p. with different doses of anti-N-PCT-(44–57) (50, 100 and 200 μg/kg of body weight) 1 h before LPS (15 mg/kg of body weight, i.p.). As mentioned above, the survival rate is 0 % after 48 h in the group of rats given this dose of LPS alone. In contrast, in the groups of rats that received anti-N-PCT-(44–57) at doses of 50, 100 or 200 μg/kg of body weight, survival rates were up to 35, 75 and 85 % respectively (Figure 3A). The Kaplan–Meier survival curve revealed a significantly better clinical outcome in rats with anti-N-PCT-(44–57) below the median compared with those with LPS above the median (P < 0.01). All doses prevented mortality and the development of clinical manifestations of endotoxin toxicity. However, in contrast with lower doses (results not shown), the highest dose of anti-N-PCT-(44–57) reversed the initial and the late progressive hypothermia induced by the LD100 of LPS (Figure 3B). At 3 days after the administration of 200 μg of anti-N-PCT-(44–57) we observed an improvement in the clinical state of surviving rats: they became more active and no longer had clinical signs of systemic inflammation. Furthermore, no late death occurred over 3 weeks, suggesting that anti-N-PCT-(44–57) did not merely delay the onset of LPS lethality, but provided lasting protection. No toxic effects of anti-N-PCT-(44–57) were observed in rats only given anti-N-PCT-(44–57), including changes in core body temperature, diarrhoea, piloerection and depressed spontaneous activity. Thus we used the dose of 200 μg of anti-N-PCT-(44–57) in the next experiments.
Next, we examined the effects of a mouse anti-(human N-PCT) monoclonal antibody (100 μg/kg of body weight, i.p.) on LPS-induced lethality to confirm further the specificity of the polyclonal antibody used in these experiments. A single dose of monoclonal anti-N-PCT given 1 h before an LD100 of LPS significantly increased the survival rate of rats compared with the LPS group (Kaplan–Meier test, \( P < 0.01 \)). In comparison with animals that received a similar dose of anti-N-PCT-(44–57), the rats that received monoclonal anti-N-PCT 1 h before LPS had a mortality of 10% at 12 h, 35% at 24 h and 55% at 48 h (Figure 3A). Similar to anti-N-PCT-(44–57), we observed an improvement in the clinical state in surviving rats after monoclonal anti-N-PCT treatment. Taken together, these findings support the specificity of the polyclonal antibody used and indicate that anti-N-PCT consistently prevented rats from LPS-induced death. As monoclonal antibodies are significantly more expensive and are only available in limited quantities, anti-N-PCT-(44–57) at a dose of 200 μg/kg of body weight was used for subsequent experiments.

### A slight delay in anti-N-PCT-(44–57) administration still prevents endotoxaemic lethality

To investigate further whether anti-N-PCT-(44–57) still protect rats from death, rats were administered with a single dose of anti-N-PCT-(44–57) (200 μg/kg of body weight) 2 h after the onset of endotoxaemia, a time immediately after the early peak in plasma TNF-α (which occurs within the 1–2 h after LPS challenge), and at which clinical signs of LPS-induced toxicity were already evident. In comparison with prophylactic treatment, the animals that received anti-N-PCT-(44–57) 2 h after LPS had a mortality of 10% at 12 h, 40% at 24 h and 45% at 48 h (Figure 4A). This level of protection was maintained throughout the course of the study. In a parallel set of endotoxaemic rats, survival was monitored for up to 3 weeks (results not shown). We found that all of the surviving animals remained alive. Furthermore, non-surviving animals injected with anti-N-PCT-(44–57) before or after LPS had almost three times the mean survival time until death in comparison with control LPS-treated rats (ANOVA, \( F_{2,35} = 44.0, P < 0.001 \); anti-N-PCT-(44–57) + LPS and LPS + anti-N-PCT-(44–57) compared with LPS using a Student–Newman–Keuls post-hoc test, \( P < 0.001 \) each). In addition, the mean survival time in rats pre-treated with anti-N-PCT-(44–57) was significantly higher than that observed when anti-N-PCT-(44–57) was administered after LPS (\( P < 0.01 \)), suggesting that immunoneutralization of N-PCT-(44–57) protects from lethal endotoxaemia by down-regulating both the early and the progressive and uncontrolled overproduction of pro-inflammatory cytokines.

### Effect of anti-N-PCT-(44–57) on LPS-induced changes in the levels of ProCT and CT

As expected, peripheral administration of LPS (15 mg/kg of body weight, i.p.) induced an increase in plasma ProCT compared with the effects of PBS-treated controls after 1, 3, 6, 12 and 24 h (Figure 5A). At these time points, plasma ProCT levels in control anti-N-PCT/PBS- or NRA/PBS-treated animals were extremely low or...
Anti-N-PCT-(44–57) pre-treatment reduces LPS-induced hypothalamic Calc-I gene expression and ProCT production in plasma and hypothalamus

Rats were injected with a single dose of anti-N-PCT-(44–57) (200 μg/kg of body weight, i.p.) as indicated, followed 1 h later by an LD<sub>100</sub> dose of LPS (15 mg/kg of body weight, i.p.). Plasma and protein extract from the hypothalamus were collected at different time points after endotoxin injection, and the Calc-I gene, ProCT and CT contents were determined, as described in the Material and methods section. Because there was no significant difference between NRA + PBS and anti-N-PCT + PBS, these results were integrated in the same curve. Results are presented as means ± S.E.M. (n = 8 rats at 1, 3 and 6 h; n = 7 at 12 h; and n = 6 at 24 h). **P < 0.01 and ***P < 0.001 compared with NRA + LPS-challenged animals (ANOVA, followed by a Student–Newman–Keuls post-hoc test).

**Figure 5** Anti-N-PCT-(44–57) pre-treatment reduces LPS-induced hypothalamic Calc-I gene expression and ProCT production in plasma and hypothalamus

Effect of anti-N-PCT-(44–57) on LPS-induced hypothalamic Calc-I gene expression and ProCT production

As shown in Figure 5(C), LPS induced significant Calc-I expression in the hypothalamus (ANOVA F<sub>2,20</sub> = 74.5, P < 0.001) compared with PBS treatment. The post-hoc test revealed that relative Calc-I mRNA expression was significantly increased beginning at 1 h, reaching a maximum at 3 h and then decreasing at 6, 12 and 24 h. In normal rats, the i.p. administration of anti-N-PCT-(44–57) alone caused no evident adverse effects (results not shown). We next evaluated the plasma production of CT during endotoxaemia. LPS injection caused a slight but significant increase in plasma CT concentrations at 1 h after injection, which was significantly higher than circulating CT levels in control animals (NRA + LPS compared with anti-N-PCT-(44–57) + PBS using a Student–Newman–Keuls post-hoc test, P < 0.01; Figure 5B). After this time point, LPS injection did not induce sustained changes in plasma CT levels (ANOVA F<sub>2,134</sub> = 0.89, P = 0.3). The increase in CT levels observed at 1 h was not affected by administration of anti-N-PCT-(44–57) to the LPS-treated group, in which the circulating levels of CT were similar to the values obtained in the animals treated with NRA + LPS. These findings suggest that CT is not involved in the protective effect of anti-N-PCT-(44–57) in this experimental model of lethal endotoxaemia.
Effects of pre-treatment with anti-N-PCT-(44–57) on LPS-induced release of TNF-α, IL-1β, IL-10 and ACTH in plasma

Rats were injected i.p. with an LD_{100} dose of LPS (15 mg/kg of body weight). Anti-N-PCT-(44–57) (200 μg/kg of body weight, i.p.) was administered 1 h before challenge with LPS. Circulating levels of TNF-α, IL-1β, IL-10 and ACTH were measured by ELISA in plasma from rats killed at 0, 1, 3, 6, 12 and 24 h after LPS injection. Because there was no significant difference between NRA + PBS and anti-N-PCT + PBS, these results were integrated in the same curve. Results are presented as means ± S.E.M. (n = 8 rats at 1, 3 and 6 h; n = 7 at 12 h and n = 6 at 24 h). *P < 0.05, **P < 0.01 and ***P < 0.001 compared with NRA + LPS-treated animals (ANOVA, followed by a Student–Newman–Keuls post-hoc test).

of anti-N-PCT-(44–57) (200 μg/kg of body weight, i.p.) 1 h before LPS significantly decreased both Ccalc-1 gene expression and ProCT production in the hypothalamus from 1 to 12 h (P < 0.01 compared with NRA + LPS-treated animals) (Figures 5C and 5D). In normal rats, the i.p. administration of anti-N-PCT-(44–57) alone caused no evident adverse effects and no changes in ProCT levels at any time (results not shown). These findings support the hypothesis that LPS-induced increase in Ccalc-1 gene expression and release of ProCT plays a role in the regulation of the neuroimmune response.

Treatment with anti-N-PCT-(44–57) reduces systemic inflammatory responses in endotoxaemic rats

We next evaluated the effect of anti-N-PCT-(44–57) on the production of pro- and anti-inflammatory mediators that are mechanistically linked to endotoxaemia. The administration of anti-N-PCT-(44–57) attenuated the LPS-induced increases in plasma TNF-α and IL-1β (Figures 6A and 6B). In addition, anti-N-PCT-(44–57) administration increased the systemic levels of the anti-inflammatory factors IL-10 and ACTH (Figures 6C and 6D), which have been shown to be protective in endotoxic shock [35,36]. We found that LPS injection significantly increased plasma TNF-α and IL-1β levels compared with PBS-treated rats (ANOVA F_{2,20} = 15.8 and F_{2,20} = 40.7, P < 0.001 respectively). Post-hoc analysis indicated that levels of TNF-α and IL-1β increased to a maximum at 1 and 3 h respectively (P < 0.001 each compared with PBS). The decrease in TNF-α plasma levels began after this time. On the other hand, plasma IL-1β levels were sustained for 6 h and then decreased at 12 h after LPS challenge. As shown in Figures 6(A) and 6(B), the administration of anti-N-PCT-(44–57) significantly inhibited the LPS-induced increase in plasma TNF-α and IL-1β levels from 1 to 6 h, and both plasma TNF-α and IL-1β levels were similar to the levels of PBS-treated rats at 12 h. In normal rats, the i.p. administration of anti-N-PCT-(44–57) alone caused no evident adverse effects and no changes in cytokine levels at any time (results not shown). Furthermore, the plasma levels of these cytokines were not detected in the PBS-administered rats. We also found that LPS increased the systemic levels of the anti-inflammatory cytokine IL-10 (ANOVA F_{2,20} = 69.5, P < 0.001) (Figure 6C). Compared with the expression of TNF-α and IL-1β, IL-10 levels increased more gradually, peaking at 6 h (P < 0.001 compared with vehicle) and then decreased gradually, but still remained significantly
Table 1  Effect of anti-N-PCT on plasma cytokine, ACTH, CT and ProCT levels 3 and 7 days after LPS administration in long-term surviving animals

Animals were pre-treated i.p. with either control NRA (IgG) or anti-N-PCT (200 μg/kg of body weight) 1 h before an LD₃₀₀ dose of LPS (15 mg/kg of body weight). Values are means ± S.E.M. for four rats per group. The groups of animals treated with NRA + LPS or PBS + LPS were not included because no animals survived at these time points. * P < 0.05 compared with control basal levels (zero time).

<table>
<thead>
<tr>
<th>Mediator</th>
<th>Time (h) after LPS treatment</th>
<th>Anti-N-PCT-(44–57) + PBS</th>
<th>Anti-N-PCT-(44–57) + LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α (ng/ml)</td>
<td>0</td>
<td>≤ 0.015</td>
<td>≤ 0.015</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>≤ 0.015</td>
<td>≤ 0.015</td>
</tr>
<tr>
<td></td>
<td>168</td>
<td>≤ 0.015</td>
<td>≤ 0.015</td>
</tr>
<tr>
<td>IL-1β (ng/ml)</td>
<td>0</td>
<td>≤ 0.005</td>
<td>≤ 0.005</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>≤ 0.005</td>
<td>0.08 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>168</td>
<td>≤ 0.005</td>
<td>≤ 0.005</td>
</tr>
<tr>
<td>IL-10 (ng/ml)</td>
<td>0</td>
<td>≤ 0.010</td>
<td>≤ 0.010</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>≤ 0.010</td>
<td>0.11 ± 0.02*</td>
</tr>
<tr>
<td></td>
<td>168</td>
<td>≤ 0.010</td>
<td>0.07 ± 0.02*</td>
</tr>
<tr>
<td>ACTH (ng/ml)</td>
<td>0</td>
<td>0.40 ± 0.12</td>
<td>0.38 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.57 ± 0.12</td>
<td>1.68 ± 0.40*</td>
</tr>
<tr>
<td></td>
<td>168</td>
<td>0.48 ± 0.10</td>
<td>0.77 ± 0.21*</td>
</tr>
<tr>
<td>CT (ng/ml)</td>
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<td>0.39 ± 0.12</td>
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<td>72</td>
<td>0.41 ± 0.10</td>
<td>0.46 ± 0.16</td>
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<tr>
<td></td>
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<td>0.43 ± 0.18</td>
<td>0.41 ± 0.12</td>
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<tr>
<td>ProCT (ng/ml)</td>
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<td>≤ 0.10</td>
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<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td>168</td>
<td>≤ 0.10</td>
<td>0.13 ± 0.05</td>
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</tbody>
</table>

elevated at 12 and 24 h after LPS and until animals died (P < 0.001 compared with vehicle). The plasma levels of this cytokine were not detected in the PBS-treated rats. Interestingly, we found that anti-N-PCT-(44–57) pre-treatment increased the systemic levels of IL-10 at 6 h (ANOVA F₁,₂₃ = 89.8, P < 0.05), 12 h (ANOVA F₁,₂₃ = 110.4, P < 0.001) and 24 h (ANOVA F₁,₂₀ = 68.0, P < 0.01) in LPS-treated rats. These results suggest that up-regulation of IL-10, which has been shown to be protective in murine models of endotoxic shock [37], is involved in anti-N-PCT-induced survival.

Because the hypothalamic–pituitary–adrenocortical axis is mechanistically linked with neuroimmune responses to acute endotoxaemia and ACTH can protect against the lethal effects of endotoxin [36], we next examined the role of ACTH in mediating the protective effect of anti-N-PCT-(44–57) on LPS-induced lethality. As shown in Figure 6(D), i.p. injection of a high dose of LPS resulted in a significant increase in the levels of ACTH in plasma compared with controls (ANOVA F₁,₂₀ = 27.3, P < 0.001), reaching a peak at 1 h. Levels then decreased after 3 h and fell at 24 h. Interestingly, treatment with anti-N-PCT-(44–57) resulted in an increase in plasma ACTH levels that was significant from 12 to 24 h after LPS challenge (P < 0.05). In normal rats, the i.p. administration of anti-N-PCT-(44–57) alone caused no evident adverse effects and no changes in plasma ACTH levels at any time (results not shown). Taken together, these findings suggest that the up-regulation of ACTH in concert with IL-10 plays a relevant role in anti-N-PCT-(44–57)-induced survival in this murine model of lethal endotoxaemia.

Neutralization of circulating N-PCT normalizes pro-inflammatory cytokine levels without removal of positive stimulation of ACTH in long-term survivors

We next evaluated plasma levels in long-term survivors at 3 and 7 days after the LPS challenge. We found that immunoneutralization of N-PCT not only protects animals from lethal endotoxaemia, but normalizes ProCT, TNF-α and IL-1β levels in long-term survivors without the removal of positive stimulation of the anti-inflammatory mediators IL-10 and ACTH (Table 1). In a parallel set of endotoxaemic rats, survival was monitored for up to 3 weeks. We found no significant differences in food intake or ProCT, TNF-α, IL-1β and IL-10 levels when compared with control animals (results not shown). These findings suggest that up-regulation of late IL-10 and ACTH production during lethal endotoxaemia plays a significant role in the long-term survival observed in animals pre-treated with anti-N-PCT-(44–57).


DISCUSSION

The present paper deals with a very important and relevant clinical issue: septic-shock-induced morbidity and mortality. In contrast with the transient increase in these pro-inflammatory cytokines, for which immunoneutralization has been disappointing [3,38], our present findings confirm and expand the importance of a targeted component of ProCT, N-PCT, as a therapy for sepsis and systemic inflammation [21,22]. The pathogenesis of lethal sepsis remains obscure, but is associated with dysregulated production of inflammatory mediators. TNF-α and IL-1β are important mediators of shock; both induce metabolic changes that are similar to those caused by endotoxin or live Gram-negative bacteria [39,40]. Most studies have shown an association between TNF-α levels, severity of shock and death [41]. Unlike these pro-inflammatory cytokines, whose appearance in the systemic circulation is transient, ProCT and N-PCT concentrations remain increased for the duration of the inflammatory stimulus [13,42]. This feature of ProCT makes it a more useful indicator for outcome prediction, as well as a potential target for therapeutic blockade.

The first main finding of present study was that N-PCT neutralization by means of specific anti-PCT antibodies against a highly conserved segment of N-PCT (amino acids 44–57) reversed N-PCT-induced inhibition of food intake in a time- and dose-dependent manner when injected into the lateral brain ventricle. These results are very important because they confirm that the N-terminal peptide of ProCT is, by itself, toxic within the central nervous system and causes systemic effects [23,24]. Furthermore, they also confirm the in vivo specificity of the antibody used in the present experiments. The second main finding of the present study was that the massive increase in circulating early pro-inflammatory cytokines after an LD100 of LPS was abolished by immunoneutralization of endogenous N-PCT and was accompanied by a significant increase in survival. Thus these results are notable in reinforcing and expanding the important role of ProCT and its component peptides in septic shock.

The results of the present study confirm that injection of a high dose of LPS (i.e. 15 mg/kg of body weight) induces a septic-shock-like state, with profound inflammatory and immunostimulatory responses both in the periphery and brain similar to pathophysiological changes found in human sepsis and septic shock. In the present study, we show that this septic-shock-like state induced by LPS correlates with disease severity and outcome, and was coupled with an early, progressive and sustained increase in plasma ProCT levels without corresponding CT secretion [14]. We also found an increased expression of the Calc-1 gene and ProCT in the hypothalamus in response to lethal endotoxaemia. In addition, we observed a very high induction in a set of mediators that have been implicated in inflammatory processes (i.e. IL-1β, TNF-α and IL-10). However, and in contrast with ProCT, TNF-α and IL-1β increase and decline early during endotoxaemia. LPS also activates the hypothalamic–pituitary–adrenal axis and increases blood concentrations of ACTH. Not surprisingly, and in agreement with other authors [15,19], LPS injection did not induce sustained changes in plasma CT levels.

It has been shown that ProCT synthesis may be induced either directly via endotoxin or indirectly via a humoral or cell-mediated host response [14,43]. Because the increase in ProCT was preceded by a release of TNF-α and IL-1β, these findings suggest that ProCT could be a secondary mediator which may augment and amplify rather than initiate the response to infectious stimuli [44]. Furthermore, previous studies indicate that the sustained elevation of ProCT in sepsis allows its neutralization to be effective during the course of this disorder [45]. Administration of ProCT to hamsters with sepsis and peritonitis doubled their death rate. Conversely, immunoneutralization with ProCT-reactive antiserum increased the survival in hamsters and pigs with sepsis [22,33], even when the ProCT-reactive antiserum was administered after the animals became moribund [21]. In addition, only the injection of human ProCT to animals with sepsis worsened the outcome, whereas in healthy animals the administration of similar doses of ProCT did not have any detrimental effects. These findings suggest that the biological activity of ProCT also appears to depend on the inflammatory status of the organism. However, the mechanism of action and the type of interaction through which ProCT interferes with immunological function has not been elucidated. The present findings support the functional significance of ProCT in terms of immune response and indicate that its N-terminal peptide plays a key immunoregulatory role in both the early and later course of severe sepsis.

Because the massive increase in circulating ProCT that persisted until animals died after LPS challenge, we analysed the effects of passive immunoneutralization of endogenous N-PCT on lethality induced by an LD100 of LPS. Ours results show that anti-N-PCT-(44–57) increased survival in endotoxaemic rats. Furthermore, anti-N-PCT-(44–57) delays the onset of mortality in non-surviving animals and provides lasting protection. In addition to preventing mortality when anti-N-PCT-(44–57) was given before LPS, anti-N-PCT-(44–57) also diminished mortality when it was administered after the acute-phase cytokine responses had peaked and resolved. In this case, the reduction in mortality and the mean time survival were significantly less pronounced than in the prophylactic study, suggesting that some of the effects of anti-N-PCT-(44–57) occur as proximal events in the course of sepsis. In both early and late therapy, the only animals to survive at the predetermined time of killing were those that had received immunoneutralization therapy, suggesting that N-PCT is integral both to the
host response and to the ultimate outcome. Because treatment with anti-N-PCT-(44–57) began after the early acute-phase response to the endotoxin, the significant protection conveyed by anti-N-PCT-(44–57) suggests that it might target late-acting mediators of lethal systemic inflammation. These results support and extend the previous findings in pigs with sepsis showing that N-PCT immunoneutralization plays an immunoregulatory role in both the early and later course of sepsis [21,22]. The fact that ProCT concentrations remain increased longer than these pro-inflammatory cytokines may also be important. We also show that N-PCT immunoneutralization with an anti-N-PCT monoclonal antibody prevented LPS-induced lethality in rats. We found no significant differences in mortality between polyclonal anti-N-PCT-(44–57) and monoclonal anti-N-PCT antibodies. Such findings are similar to what have been reported in other animal studies using various types of immune neutralization against endotoxin, TNF-α, and IL-1β [39].

Of physiological relevance is the observation that neutralization of N-PCT down-regulates the expression of the *Calc-1* gene and the production of ProCT in the hypothalamus, a key regulatory area involved in the pathways and mechanisms of immune–brain interaction during septic shock [46]. It has been postulated that the presence of microbial infection-specific response elements or cytokines (e.g. TNF-α and IL-6) in the *Calc-1* gene promoter might override the tissue-selective expression pattern upon a specific stimulus, thereby significantly influencing the response to systemic inflammation [43]. In the present study, we found that administration of endotoxin resulted in a substantial increase in the expression of *Calc-1* as well as a time-dependent production of ProCT in the hypothalamus, which is consistent with a co-ordinated autonomic and behavioural response to the LPS challenge. However, these findings do not demonstrate that this particular expression of the *Calc-1* gene, and subsequent ProCT production, in the hypothalamus is causally linked to N-PCT and the septic symptoms displayed by the animals. Previous results have shown that i.c.v. administration of N-PCT causes sickness-type behaviours similar to that induced by peripheral injection of bacterial endotoxin in rats (i.e. fever, cachexia and anorexia) [23]. These sickness symptoms were mediated by activation of prostaglandin-dependent pathways within the hypothalamus [24], where LPS and cytokines mediate effects on energy balance [47]. Recent in vitro studies in COS-7 cells suggest that ProCT has bioactivity at CT receptor family complexes and is a potential mediator in sepsis [48]. Therefore it is tempting to speculate that N-PCT may activate CT/CT-like receptors, which are abundantly expressed in brain regions of primary importance in the regulation of energy homoeostasis [49]. Taken together, these observations suggest that N-PCT may act as an important mediator of neuroimmune actions and could serve as a circulating afferent signal for the activation of inflammatory responses to sepsis. However, it remains unclear whether circulating N-PCT is functional in the brain and, if so, whether it activates CT/CT-like receptors.

Regulation of innate immune responses is a delicate balancing act, and dysregulated innate immune reactions, by either default or excess, have dramatic consequences for the infected host, as seen in severe sepsis. Thus the mechanisms underlying anti-N-PCT-(44–57)-mediated protection against lethal endotoxaemia may be multiple, ranging from increasing production of anti-inflammatory mediators (e.g. IL-10 and ACTH) to inhibition of LPS-induced release of pro-inflammatory cytokines, such as TNF-α and IL-1β. Our present results show that immunoneutralization of N-PCT is able to reduce the exacerbated humoral immune responses induced by an LD<sub>50</sub> dose of endotoxin in rats. In most animals in our present study, the plasma levels of TNF-α and IL-1β decreased toward normal levels within the first day in rats treated with anti-N-PCT-(44–57), despite a persisting critical clinical condition. In contrast with TNF-α and IL-1β, the plasma levels of IL-10 were not modified by anti-N-PCT and remained significantly increased until clinical recovery. Because N-PCT is specifically induced by LPS and TNF-α and it has been shown that N-PCT modulates LPS-induced CD11b up-regulation in polymorphonuclear leucocytes [20], the results of the present study suggest that, once N-PCT is released into the bloodstream, it may act to amplify the LPS-induced shock response and synergistically increase endotoxin-induced animal lethality.

Bacterial endotoxin has been reported previously to profoundly activate the hypothalamic–pituitary–adrenal axis, resulting in elevated ACTH secretion that may serve an important role as part of the inhibitory feedback mechanisms on the activated immune system [35]. In the present study, we found that plasma levels of ACTH are maintained after anti-N-PCT-(44–57) during the first 24 h, suggesting that ACTH could play an important role in survival of endotoxaemic animals treated with anti-N-PCT. Furthermore, other additional mechanisms may be involved in anti-N-PCT-(44–57)-mediated protection against lethal endotoxaemia.

The present results also indicate that anti-N-PCT-(44–57) increases survival time in non-surviving rats and plays a direct role in long-term survival. These effects correlate with normalization in TNF-α and IL-1β levels. An additional mechanism to explain the protective effect of anti-N-PCT-(44–57) in long-term survivors is the increase in the delayed production of counter-regulatory anti-inflammatory mediators such as IL-10 and ACTH. In this context, the balance between TNF-α and IL-10 is important for immune homoeostasis maintenance. Excessive production of TNF-α contributes to an overwhelming inflammatory response and tissue damage [50]. However, an increase in TNF-α is counterbalanced by the
simultaneous synthesis of an anti-inflammatory cytokine, IL-10, which suppresses the production of many activating and regulatory mediators [51]. In the present study, we found that, in contrast with endotoxemic animals, the condition of all of which progressively worsened and finally died, physiological parameters and IL-10 levels of anti-N-PCT-(44–57)-treated animals improved or stabilized. These findings suggest that N-PCT could play an immunoregulatory role by regulating the balance between TNF-α and IL-10 at this late stage, where the majority of rats surviving LPS alone and LPS + anti-N-PCT treatment still had symptoms. Interestingly, ACTH levels are significantly increased in long-term surviving animals treated with anti-N-PCT-(44–57). These results suggest that the augmentation by anti-N-PCT-(44–57) of the secretion of anti-inflammatory mediators most probably shifts the cytokine balance in the anti-inflammatory direction.

There are, however, potential limitations to the present study. First of all, although the animal model we used is comparable with the multiple organ failure that occurs in humans with pre-terminal sepsis [1], mortality rates in sepsis are highly dependent on multiple host- and pathogen-related factors (i.e. co-morbidities of the host and virulence of the bacteria, among others). Thus further information on the mechanisms by which N-PCT acts are necessary to understand where this peptide fits in the complex network of events that occurs in septic shock. Secondly, there is a limitation about the specificity of the antibody used. Although the antibodies used are directed against the entire molecule (monoclonal antibody) or a highly conserved amino acid sequence [polyclonal anti-N-PCT-(44–57)] of the known N-PCT molecule, N-PCT is an integral part of the native molecule ProCT, and it is not possible to distinguish the role of N-PCT from its immediate precursor ProCT, as long as it is potentially able to bind to this molecule. The present results suggest that the antibody used in our study reacts specifically with N-PCT and support the notion that immunoneutralization of N-PCT with specific neutralizing antibodies might be beneficial in improving the unfavourable prognosis of septic shock.

In summary, in the present study, we have shown that specific immunoneutralization of N-PCT can inhibit LPS-induced lethality in rats. The ability of anti-N-PCT-(44–57) to inhibit the production, release or action of pro-inflammatory cytokines partially accounts for this protective effect of anti-N-PCT-(44–57). The present findings suggest that N-PCT, besides being a reliable marker of severity and mortality, plays a crucial role in septic shock and directly affects the outcome by regulating the production of pro- and anti-inflammatory factors crucial for the development of this syndrome. In agreement with this hypothesis, our preliminary results in a model of sepsis induced by caecal ligation and puncture, a clinically relevant model for human sepsis because it causes lethal peritonitis produced by polymicrobial infection, indicate that treatment with anti-N-PCT started after the induction of sepsis, at a time when rats have clear signs of sepsis, significantly improved survival (E. Tavares and F. J. Miñano, unpublished work). These experimental results provide strong support for the concept of ProCT-targeted therapy for septic shock.

**AUTHOR CONTRIBUTION**

Both authors contributed to the conception and design of the study, analysis and interpretation of data, and to drafting or critical revision of the article. Francisco Miñano wrote the paper.

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Aminoprocyclotin and septic shock


