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Immunoneutralization of the Aminoprocaltitonin Peptide of Procalcitonin Protects Rats from Lethal Endotoxemia: Neuroendocrine and Systemic Studies

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Abbreviations: aCSF, artificial cerebrospinal fluid; ACTH, adrenocorticotrophic hormone; IL, interleukin; icv, intracerebroventricular; LPS, lipopolysaccharide; NRA, normal rabbit antibody; N-PCT, aminoprocaltitonin peptide; PBS, phosphate buffer saline; RT-PCR, real-time polymerase chain reaction; TNF, tumor necrosis factor

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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 lipopolysaccharide (LPS) and proinflammatory cytokines, particularly tumor necrosis factor (TNF)- α and interleukin (IL)-1 β . Severity and mortality of sepsis have also been associated with high concentrations of aminoprocaltitonin (N-PCT), a 57-amino acid neuroendocrine peptide derived from procalcitonin. Prior 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 further explore 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-PCT₄₄₋₅₇). 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-PCT₁₋₅₇. We next further explored the therapeutic potential of anti-N-PCT₄₄₋₅₇ in a rat model of lethal endotoxemia and determined how this immunoneutralization affected LPS-induced lethality and cytokine production. We showed that this specific antibody inhibits LPS-induced early release of TNF- α and IL-1 β and increased survival; even if treatment began after cytokine response has occurred. Also, anti-N-PCT₄₄₋₅₇ may increase long-term survival in LPS-treated rats by up-regulating the late production of counterregulatory anti-inflammatory mediators such as ACTH and IL-10. These data support N-PCT as a proinflammatory factor in both the early and the late stages of lethal endotoxemia and suggest 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 lipopolysaccharide (LPS) endotoxin, which triggers the release of proinflammatory cytokines, including tumor necrosis factor (TNF)- α and interleukin (IL)-1 β [1]. Counterregulatory anti-inflammatory mediators, such as glucocorticoids and IL-10, normally suppress proinflammatory 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 the septic shock syndrome, and lethal endotoxemia has been extensively used as an experimental model of septic shock [4]. Significant advances have been made in understanding the role of proinflammatory mediators in the pathogenesis of sepsis, but effective therapies have not yet entered clinical practice [5]. A major difficulty in developing therapeutics that target proinflammatory cytokines (e.g., TNF- α and IL-1 β) 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-1 β that 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 calcitonin (CT) peptide family derived from Calc-1 gene, including procalcitonin (ProCT) and amino-procalcitonin (N-PCT) [7]. N-PCT was first described in rats as a 57-amino acid neuroendocrine peptide derived from the amino-terminal half of ProCT [8]. Synthesis of N-PCT is complex and starts with the translation of a 141 amino acid pre-prohormone (pre-procalcitonin, PPCT) 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: carboxypeptide-1 (CT:CCP-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 the human and rodent [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 these peptides circulate in low concentrations as free peptides in the serum of normal persons [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 of 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 septic humans [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-I 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 another one is through cell-mediated host response mediated by proinflammatory cytokines (in particular TNF- α) [11, 19]. Furthermore, it has been shown that N-PCT carries leukocyte 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 septic pigs and greatly increased their short-term survival [21-22]. Recent *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 behaviors 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 proinflammatory cytokines (e.g., IL-1 β , 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 further explore 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 human and rat. We test here the effects of this antibody in a model of lethal endotoxemia 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 endotoxemic rats with this specific antibody against N-PCT attenuated morbidity and markedly increased their survival by down-regulating the innate inflammatory response.

MATERIALS AND METHODS

Reagents

The polyclonal rabbit anti-human calcitonin precursor used in our studies was synthesized by AbD Serotec (Product Code: 1720-9670; Oxford, UK). The immunogen used was a synthetic peptide corresponding to amino acids 69-82 (⁶⁹QERE⁸²GS SLDSPRS⁸²) of human pre-procalcitonin (PPCT), the native molecule of N-PCT. According to the BLAST protein database search program (SIB BLAST network service), this sequence corresponds to a specific and highly conserved amino-terminal segment of human N-PCT (amino acids 44-57) and differs from that of the rat sequence by only one amino acid (⁴⁴QEA⁵⁷EGS SLDSPRS⁵⁷). Furthermore, it does not present alignments with any other member of the CT/CT gene related peptide family.

For central studies, purified human N-PCT (¹APFRSAL ESSPADP ATLSEDE ARLLAA LVQDYVQ MKASELE QEQEREG SLDSPRS⁵⁷) 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, and was purchased from Bachem (Bubendorf, Switzerland). Reverse-phase high performance liquid chromatography (HPLC), amino acid sequencing, and mass spectrometry ascertained purity of the peptide. N-PCT and anti-N-PCT₄₄₋₅₇ were dissolved in a microfiltered and sterile solution of rat artificial cerebrospinal fluid (aCSF; Harvard Apparatus, Inc., Holliston, MA, USA). Heat-inactivated N-PCT₄₄₋₅₇ (90°C, 1h) and normal IgG rabbit antibody (NRA) Sigma-Aldrich, St. Louis, MO, USA) were used as controls.

For peripheral studies, lethal endotoxemia was induced by bacterial lipopolysaccharide (LPS, Escherichia coli, serotype 0111:B4; Sigma Chemical Co., St. Louis, MO, USA) was dissolved in phosphate buffered saline (PBS) and dispersed by brief sonication before intraperitoneal (ip) injection. In these immunization studies, anti-N-PCT₄₄₋₅₇ was dissolved in PBS and administered ip. PBS and NRA were used as controls. In some experimental groups we administered ip a mouse monoclonal ProCT antibody (6f10, Abcam Inc., Cambridge, MA, USA) to determine how this immunoneutralization affected LPS-induced lethality in comparison with a similar dose of anti-N-PCT₄₄₋₅₇. In these experiments, the immunogen used was a synthetic peptide corresponding to the entire molecule of human N-PCT (amino acids 1-57). In these experiments, an isotype goat anti-mouse IgG1 (Biogenex, San Ramon, CA, USA) was used as 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 by the limulus lysate assay (ICN Biomedicals, Costa Mesa, CA). Fresh aliquots were thawed before each experiment, briefly sonicated and warmed to 37 °C just before use.

Animals and housing

Adult male Wistar rats (225-275 g. Center for Animal Production and Research, University of Seville, Spain) weighing 225–250 g were maintained in individual cages under controlled temperature and light (lights on at 0700 h; lights off at 1900 h) with ad libitum access to tap water and a nutritionally balanced rodent diet (2014 S; Harlan Iberica, Barcelona, Spain). All experiments were performed at this ambient

temperature of 26-28 °C ($50 \pm 5\%$ humidity), rather than at 20-22 °C, an ambient temperature below the zone of thermoneutrality for rat, 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 for only 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-PCT₄₄₋₅₇. Effects of intracerebroventricular anti-N-PCT on N-PCT-induced anorexia

Before investigating the therapeutic potential of the anti-N-PCT₄₄₋₅₇ antibody 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 intracerebroventricular (icv) infusion cannulas and injections were performed essentially following standard surgical procedures [23]. Rats were anesthetized with a ketamine cocktail (100 mg/ml ketamine, 5 mg/ml xylazine, 10 mg/ml acepromazine) and 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, 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, s.c.; Metacam®, Boehringer Ingelheim España, S.A, Barcelona, Spain), a non-steroidal anti-inflammatory drug used for postoperative pain in rats [27], was administered immediately after surgery while the rats were still under anesthesia 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 wk with unrestricted access to water and food prior to the onset of the experiment.

On the test day, groups of weight-matched ($\pm 5\%$) rats ($n = 8$ for each treatment combination) were infused icv with 5 μ l of either anti-N-PCT antibody₄₄₋₅₇ (0.5, 1 and 2 μ g) or equivalent doses of control rabbit NRA, 30 min before 1 nmol/rat (5 μ g/rat) of N-PCT or an equivalent volume of control aCSF. We have previously shown that this dose of N-PCT effectively reduces food intake in ad libitum-fed rats [23-24]. Heat-inactivated N-PCT (1 nmol, icv; 90 °C, 1h) was used as control peptide. Drugs were infused by gravity flow through an injector needle lowered into the ventricular lumen, and the injection cannulas were left in place for an additional 1 min after the injection. Immediately after injections, a preweighed amount of food was presented to the animals. Cumulative food intake was measured by placing pre-weighed pellets on a top-loading balance with accuracy up to ± 0.1 g and weighing uneaten pellets at 2, 4, and 24 h after treatment. Water was available ad libitum.

Experiment 2: Murine model of lethal endotoxemia and anti-N-PCT treatment

The purpose of these experiments was to establish a dose-response after LPS. Thus, before induction of endotoxemia, each animal was anesthetized as above, and a temperature-sensitive radio transmitter (model PDT-4000; Mini-Mitter, Sunriver, OR) was implanted into the peritoneum. Core body temperature (T_b) ($\pm 0.1^\circ\text{C}$) was continuously monitored using the VitalView system (Mini-Mitter), as described elsewhere [24, 28]. Meloxicam (1 mg/kg, sc) was administered for post-operative pain control. After recovery, animals were acclimatized to the facility for at least 1 wk 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 ($\pm 5\%$). Rats were challenged in groups of ten with LPS (dose range: 1–15 mg/kg) by ip injection. Animals were observed on mortality for 7 days and feeding, movement and activity, and other clinical signs including ruffled fur, lethargy, appearance of diarrhea, and body weight loss. Any animal that appeared moribund or unresponsive to external stimuli was killed to avoid undue pain and suffering. All survival studies were conducted in a blind and random fashion. In some experiments monitoring was continued once daily for up to 3 wk. In drug testing, using our established dose of LPS (15 mg/kg) that was 100 % lethal (LD_{100}), the effect of anti-N-PCT (50, 100 and 200 $\mu\text{g/kg}$) on LPS-induced immune responses and mortality was assessed by dosing ip anti-N-PCT 1 h before LPS challenge ($n=12$ rats per group). A separate group of rats ($n=12$) received anti-N-PCT (200 $\mu\text{g/kg}$) at 2 h after LPS, a time when animals show clear signs of established sepsis, including lethargy, piloerection, diarrhea, huddling, and malaise. Rats in control and LPS groups were only given vehicle, NRA or LPS. To further observe the effects of anti-N-PCT on endotoxemia, we used the dose of 200 μg anti-N-PCT for drug screening in the next experiments. Furthermore, because N-PCT is an integral part of ProCT, we performed some additional experiments in order to ascertain the specificity of the antibody used against the fragment of N-PCT₄₄₋₅₇. For this purpose, we used a mouse IgG₁ anti-human ProCT monoclonal antibody (100 $\mu\text{g/kg}$) or an equal dose of isotype goat anti-mouse IgG₁ as control ($n=12$ rats per group).

Effects of anti-N-PCT on inflammatory mediators

To determine the effects of anti-N-PCT on plasma cytokine, ProCT, CT and adrenocorticotrophic hormone (ACTH) responses, as well as hypothalamic Calc-1 expression and ProCT production, separate groups of animals were sacrificed at different times after LPS or after injection of LPS and anti-N-PCT₄₄₋₅₇ (200 $\mu\text{g/kg}$) administered ip 1 h before LPS. Control rats received an equal of vehicle instead of anti-N-PCT₄₄₋₅₇. Because analgesics and/or anesthetics suitable for reducing pain exhibit immunomodulatory properties and interfere with the outcome of immunological experiments dealing with inflammation (i.e., endotoxemia-induced cytokine levels [29-32], no analgesics and/or anesthetics were used. Thus, for these immunological studies, rats ($n=10-30$ per group) were euthanized by carbon dioxide (CO_2) inhalation followed by cervical dislocation and assayed (as described following) at each of the following time points after LPS injection: 1, 3, 6, 12, and 24 h ($n=10$ total per group; $n=7-8$ per time point, except for 24 h). Because LPS-induced mortality by about 80% at 24 h (see Results), a separate group of rats ($n=30$ total) was used to determine the levels of mediators at this time ($n=6$).

Tissue collection

Blood was collected, by cardiac puncture immediately before cervical dislocation, into tubes containing K₂ EDTA and cooled immediately at 4 °C. The plasma was separated by centrifugation (6000g, at 4 °C for 15 min) within the next 15 min, and then frozen and stored at -80 °C until assayed for cytokine and hormone concentrations. During necropsy, and at 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 reverse transcription polymerase chain reaction (RT-PCR) or by immunoluminometry, respectively. We also studied plasma production of inflammatory mediators in long-term survivors pretreated with anti-N-PCT⁴⁴⁻⁵⁷. Because immunoneutralization of N-PCT prevents LPS-induced mortality by about 85% (see Results), a separate group of rats was used (n=12). Survivors rats were sacrificed at 3 and 7 d after injections (n= 4 per time point). Blood samples were collected and treated as described above for determination of cytokines and hormones.

Expression of Calc-1 gene in hypothalamic samples

It has been shown that Calc-1 gene is responsible for 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 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. Calc-1 gene expression was determined by real-time polymerase chain reaction (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 dry ice and immediately frozen by immersion in liquid nitrogen. The frozen tissue was ground with mortar and pestle under nitrogen. Total RNA from rat hypothalamus was extracted using the TriPure Isolation Reagent® (Roche Applied Science, Indianapolis, USA) according to the manufacturer's instructions. Total RNA was quantified by Quant-It RiboGreen RNA Reagent (Molecular Probes). cDNA was synthesized by AffinityScript QPCR cDNA Synthesis Kit (Stratagene, La Jolla, CA, USA). Oligonucleotide primers for RT-PCR were designed using Beacon Designer Software (Premier Biosoft, Palo Alto, CA), according to the rat Calc-1 sequence NM_017338 (National Center for Biotechnology Information database). The primers for RT-PCR were: forward 5'-CGCTGCCCAGATCAAGAGTC-3'; reverse 5'-AGATGCTGACAACCAGGAAAGG-3' (Eurogentec S.A., Liege, BE). In parallel, we analyzed β_2 -microglobulin mRNA expression using the Mouse Endogenous Control Panel Kit (TATAA Biocenter AB, Göteborg, Sweden), as an internal control for normalization. The cDNA templates were denatured for 10 min at 95 °C and then amplified using 40 cycles of 30 sec at 95 °C, 1 min at 60 °C, 30 sec at 72 °C and for dissociation curve, 1 min at 95 °C, 30 sec at 60 °C and 30 sec at 95 °C. Each sample was amplified using a 25 ml of Brilliant SYBR Green QPCR Master Mix (Mx3005P

Instruments) and 0.15 mM of each oligonucleotide in an Mx3005P Real-Time PCR System (Stratagene, La Jolla, CA, USA). The level of each specific cDNA was quantified in the exponential phase of PCR product accumulation and normalized by the level of β_2 -microglobulin expression in each individual sample. Each sample of cDNA was measured by triplicate. PCR amplicons were confirmed to be specific by size and melting curve analysis.

ProCT-like immunoreactivity in plasma and hypothalamus

Plasma and hypothalamic samples were assayed for ProCT using an immunochemiluminescent, two-monoclonal antiserum sandwich assay (BRAHMS Diagnostica), as previously described in septic hamsters [33] and rats [28]. Hypothalamic samples were placed in 1N 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; Bio-Rad Laboratories, Madrid, Spain). Hypothalamic supernatant and blood plasma were analyzed 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 (coefficient of variation < 15 %). This assay has a detection limit of 0.1 ng/ml. The intra- and inter- assay coefficients of variation 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, Inc., IL, USA), IL-1 β (R&D Systems Europe, Abingdon, UK), IL-10 (IBL, Hamburg, Germany), adrenocorticotrophic hormone (ACTH) (Phoenix Pharmaceuticals, Belmont, CA, USA) and CT (Phoenix Pharmaceutical Inc., Belmont, CA). Plasma cytokine levels in rats were measured 1, 3, 6, 12, and 24 h after LPS (n = 6-8 rats per time point), as previously described [28]. The detection limit were 15 pg/ml for TNF- α , 5 pg/ml for IL-1 β , 10 pg/ml for IL-10, and 0.36 ng/ml for CT. The intra- and inter- assay coefficients of variation were below 5%. Plasma ACTH was extracted using a 1% trifluoroacetic acid buffer and C-18 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 EIA in assay buffer. The range of detection for the ACTH kit was 0–25 ng. All plasma samples were analyzed in duplicate and repeated freeze-thaw cycles for reagents, and samples were avoided

Statistical analysis

Data were expressed as the mean \pm SEM. Statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by the 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

Intracerebroventricular administration of anti-N-PCT₄₄₋₅₇ reverses N-PCT effects on food intake

In order to assess the specificity of anti-N-PCT₄₄₋₅₇, animals were injected icv with N-PCT in the presence and absence of anti-N-PCT. Consistent with previous findings in rats [23], icv 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 to the counterpart aCSF-treated controls (ANOVA $F_{3,31} = 10.9$; $P < 0.001$; aCSF + N-PCT vs. aCSF + aCSF Newman–Keuls post-hoc test, $P < 0.001$, each; see 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 (Newman–Keuls, $P > 0.05$, each).

When rats received anti-N-PCT (0.5, 1 and 2 µg/rat icv) 30 min before icv 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 N-PCT reversed N-PCT effects on food intake, so that food intake was similar to the levels of aCSF-treated animals at 2, 4 and 24 h (Newman–Keuls, $P > 0.05$, each). Thus, in the presence of this dose of anti-N-PCT, the food consumed by the N-PCT-treated group was significantly increased at 2, 4, and 24 h (anti-N-PCT + N-PCT vs. NRA + N-PCT Newman–Keuls, $P < 0.001$, each; see Figure 1B). In a different set of animals, a lower dose of anti-N-PCT (1 µg icv) was injected before N-PCT. In the presence of this dose of anti-N-PCT, the food consumed by the N-PCT-treated group was also increased at 2, 4, and 24 h (anti-N-PCT + N-PCT vs. NRA + N-PCT Newman–Keuls, 2 and 4 h: $P < 0.001$, 24 h: $P < 0.05$; see Figure 1B). A different group of animals was pre-treated with a lowest dose of anti-N-PCT (0.5 µg icv). This dose of anti-N-PCT had no significant effects on N-PCT-induced suppression of feeding at 2, 4, and 24 h (anti-N-PCT + N-PCT vs. NRA + N-PCT Newman–Keuls, $P = 0.4$, $P = 0.1$, and $P > 0.3$, respectively; see Figure 1B). Treatment of rats with anti-N-PCT + aCSF or NRA + aCSF did not affect food intake when compared to aCSF-treated rats (Figure 1 A and B).

Figure 1

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, ip) and recorded survival rates. As shown in this Figure, rats receiving 15 mg/kg of LPS all died within 48 h, but in the groups that rats were given 1, 5 and 10 mg/kg of LPS, 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 ($P < 0.01$). Based on this experiment, we chose 15 mg/kg of LPS (LD₁₀₀) for drug screening in the next experiments. This dose of LPS induces profound inflammatory and immunostimulatory responses both in the periphery 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, chromodacryorrhea and diarrhea. As previously demonstrated by others, most of the animals exhibited profound hypothermia followed by fever in an ambient temperature within the zone of thermoneutrality for rats (see Material and Methods). The animals were essentially moribund by 8 h after LPS, and showed a progressive mortality, reaching 20% at 12 h, 80% at 24 h, and 100% at 48 h (Figure 2).

Figure 2

Treatment with anti-N-PCT protects against LPS-induced toxicity

Because anti-N-PCT₄₄₋₅₇ inhibits the N-PCT-induced, we expected a protective effect in high-dose endotoxemia, a murine model for septic shock syndrome. Rats were ip dosed with different doses of anti-N-PCT (50, 100 and 200 µg/kg) and 1 h before LPS (15 mg/kg, ip), as shown in Figure 3A. As mentioned before, in the group of rats given this dose of LPS alone, the survival rate is 0 % after 48 h. In contrast, in the groups that rats received anti-N-PCT at doses of 50, 100 or 200 µg/kg, survival rates were up to 35%, 75 % and 85%, respectively. The Kaplan–Meier survival curve revealed a significantly better clinical outcome in rats with anti-N-PCT below the median compared with those with LPS above the median ($P < 0.01$). All doses prevented mortality and prevented the development of clinical manifestations of endotoxin toxicity. However, in contrast to lower doses (data not shown), the high dose of anti-N-PCT₄₄₋₅₇ reversed the initial and the late progressive hypothermia induced by this LD₁₀₀ of LPS (Figure 3B). Three days after the administration of 200 µg anti-N-PCT₄₄₋₅₇ we noted an improvement in the clinical state in surviving rats: they became more active and no longer presented clinical signs of systemic inflammation. Furthermore, no late death occurred over 3 wk suggesting that anti-N-PCT₄₄₋₅₇ did not merely delay the onset of LPS lethality, but provided lasting protection. No toxic effects of anti-N-PCT were observed in rats only given anti-N-PCT, including changes in Tb, diarrhea, piloerection, and depressed spontaneous activity. Thus, we used the dose of 200 µg anti-NPCT in the next experiments.

Figure 3

Next, we examined the effects of a mouse anti-human N-PCT monoclonal antibody (100 µg/kg, ip) on LPS-induced lethality to further confirm 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 survival rate of rats compared to LPS group (Kaplan–Meier test, $P < 0.01$). In comparison to animals that received a similar dose of anti-N-PCT₄₄₋₅₇, the rats that received monoclonal anti-N-PCT 1 h before LPS attained a mortality of 10% at 12 h, 35% at 24 h and 55% at 48 h (Figure 3A). Similar to anti-N-PCT₄₄₋₅₇ antibodies, we noted 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 prevents rats from LPS-induced death. Since monoclonal antibodies are significantly more expensive and available in limited quantities, 200µg/kg anti-N-PCT₄₄₋₅₇ was used for subsequent experiments.

Slight delay in anti-N-PCT₄₄₋₅₇ administration still prevents endotoxemic lethality

To further investigate whether anti-N-PCT still protect rats from death, rats were administered with a single dose of anti-N-PCT (200 µg/kg) 2 h after the onset of endotoxemia, a time immediately after the early peak in plasma TNF-α (which occurs within the first 1 to 2 h after LPS challenge), and at which clinical signs of LPS-induced toxicity already were evident. In comparison to prophylactic treatment, the animals that received anti-N-PCT 2 h after LPS attained 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. Furthermore, nonsurvivor animals injected with anti-N-PCT before or after LPS almost triplicate the mean survival time until death in comparison with control LPS-treated rats (ANOVA, $F_{(2,35)} = 44.0$, $P < 0.001$; Newman-Keuls: anti-N-PCT + LPS and LPS + anti-N-PCT vs. LPS, $P < 0.001$, each). In addition, the mean survival time in rats pretreated with anti-N-PCT was significantly higher than that observed when anti-N-PCT was administered after LPS ($P < 0.01$), suggesting that immunoneutralization of N-PCT protects from lethal endotoxemia by down-regulating both the early and the progressive and uncontrolled overproduction of proinflammatory cytokines.

Figure 4

Effect of anti-N-PCT₄₄₋₅₇ on LPS-induced changes in the levels of ProCT and CT

As expected, peripheral administration of LPS (15 mg/kg, ip) induced increases in plasma ProCT compared with the effects of PBS-treated controls after 1, 3, 6, 12 and 24 h (see Figure 5A). At these time points, plasma ProCT levels in control anti-N-PCT/PBS- or NRA/PBS-treated animals were extremely low or undetectable. Pretreatment with anti-N-PCT₄₄₋₅₇ (200 µg/kg), significantly attenuated LPS-induced rise in the levels of circulating ProCT at 1, 3, 6, 12, and 24 h post-treatment (ANOVA $F_{2,134} = 68.9$; $P < 0.001$; NRA/LPS vs. anti-N-PCT/LPS post hoc analysis: 1 h, $P < 0.05$; 3 h, $P < 0.01$; 6, 12 and 24 h, $P < 0.001$). In normal rats, the ip administration of anti-N-PCT alone caused no evident adverse effects (data not shown). We next evaluated the plasma production of CT during endotoxemia. 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 (ANOVA; NRA + LPS vs. anti-N-PCT + PBS Newman-Keuls, $P < 0.01$; Figure 5B). After this time point LPS injection did not induce sustained changes in plasma CT levels ($F_{2,134} = 0.3$, $P = 0.7$). The increase in CT levels observed at 1 h was not affected by administration of anti-N-PCT to the LPS-treated group, in which the circulating levels of CT were similar to the values obtained from the animals treated with NRA + LPS. These findings suggest that CT is not involved in the protective effect of anti-N-PCT in this experimental model of lethal endotoxemia.

Figure 5

Effect of anti-N-PCT₄₄₋₅₇ on LPS-induced hypothalamic Calc-1 gene expression and ProCT production

As shown in Figure 5C, LPS induced a significant Calc-1 expression in the hypothalamus ($F_{(2,20)} = 74.5$, $P < 0.001$) compared with PBS treatment. Post hoc test revealed that relative expression of Calc-1 mRNA expression was significantly increased beginning at 1 h reaching a maximum at 3 h, and then decreased at 6 h after LPS administration. Levels of Calc-1 gene expression returned to basal levels at 24 h. A small amount of Calc-1 was expressed in the PBS-treated rats. RT (-) negative control RT-PCR was performed for each sample (data not shown). As expected, peripheral administration of a high dose of LPS (15 mg/kg, ip) also induced a significant increase ($F_{(2,20)} = 33.5$, $P < 0.001$) in the hypothalamic levels of immunoreactive ProCT compared with PBS-treated animals. There was a positive correlation between this increase in hypothalamic Calc-1 signal and hypothalamic ProCT concentrations ($r = -0.89$, $P = 0.03$). As shown in Figure 5D, we found a rapid rise of ProCT within 1 h after LPS injection. Levels then rose rapidly, reaching a peak at 6-12 h, and then decreased at 24 h after LPS challenge. Pretreatment with a single dose of anti-N-PCT₄₄₋₅₇ (200 µg/kg, ip) 1 h before LPS significantly decreased both Calc-1 gene expression and ProCT production in the hypothalamus from 1 to 12 h ($P < 0.01$ vs. NRA + LPS-treated animals) (Figure 5C and D). In normal rats, the ip administration of anti-N-PCT alone caused no evident adverse effects and no changes in ProCT levels at any time (data not shown). These findings support the hypothesis that LPS-induced increase in Calc-1 gene expression and release of ProCT plays a role in the regulation of the neuroimmune response.

Treatment with anti-N-PCT reduces systemic inflammatory responses in endotoxemic rats

We next evaluated the effect of anti-N-PCT₄₄₋₅₇ on the production of pro- and anti-inflammatory mediators that are mechanistically linked to endotoxemia. The administration of anti-N-PCT₄₄₋₅₇ attenuated the LPS-induced increases in plasma TNF- α and IL-1 β (Figure 6, A and B). In addition, anti-N-PCT₄₄₋₅₇ administration increased the systemic levels of the anti-inflammatory factors IL-10 and ACTH (Figure 6, C and D), which have been shown to be protective in endotoxic shock [35-36]. We found that LPS injection significantly increased plasma TNF- α and IL-1 β levels compared to 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-1 β increased to a maximum at 1 and 3 h, respectively ($P < 0.001$ vs. PBS, each). The decrease in TNF- α plasma levels began after this time. On the other hand, plasma IL-1 β levels were sustained for 6 h and then decreased at 12 h after LPS challenge. As shown in Figure 6 A and B, the administration of anti-N-PCT₄₄₋₅₇ significantly inhibited LPS-induced increase in plasma TNF- α and IL-1 β levels from 1 to 6 h, and both plasma TNF- α and IL-1 β levels were similar to the levels of PBS-treated rats at 12 h. In normal rats, the ip administration of anti-N-PCT alone caused no evident adverse effects and no changes in cytokine levels at any time (data 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 ($F_{(2,20)} = 69.5$, $P < 0.001$) (Figure 6C). Compared with the expression of TNF- α and IL-1 β , IL-10 levels increased more gradually, peaking at 6 h ($P < 0.001$ vs. vehicle). Then, decreased gradually but still remain significantly elevated at 12

and 24 h after LPS, until animals died ($P < 0.001$ vs. vehicle). The plasma levels of this cytokine were not detected in the PBS-treated rats. Interestingly, we found that anti-N-PCT₄₄₋₅₇ pretreatment increased the systemic levels of IL-10 at 6 h (ANOVA $F_{(2,23)} = 89.8$, $P < 0.05$), 12 h ($F_{(2,20)} = 110.4$, $P < 0.001$), and 24 h ($F_{(2,20)} = 68.0$, $P < 0.01$) in LPS-treated-rats. These data 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.

Figure 6

Because hypothalamic-pituitary-adrenocortical axis is mechanistically linked to neuroimmune responses to acute endotoxemia 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 on LPS-induced lethality. As shown in Figure 6D, ip injection of a high dose of LPS resulted in a significant increase in levels of ACTH in plasma compared to control (ANOVA $F_{(2,20)} = 27.3$, $P < 0.001$); reaching the peak at 1 h. Levels then decreased after 3 h and dropped at 24 h. Interestingly, treatment with anti-N-PCT antibody 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 ip administration of anti-N-PCT alone caused no evident adverse effects and no changes in plasma ACTH levels at any time (data not shown). Taken together, these data suggest that up-regulation of ACTH in concert with IL-10 plays a relevant role in anti-N-PCT-induced survival in this murine model of lethal endotoxemia.

Neutralization of circulating N-PCT normalizes proinflammatory 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 d after the LPS challenge. We found that immunoneutralization of N-PCT not only protects animals from lethal endotoxemia if not normalizes ProCT, TNF- α and IL-1 β levels in long-term survivors without the removal of positive stimulation of the anti-inflammatory mediators IL-10 and ACTH (Table 1). These data suggest that up-regulation of late IL-10 and ACTH production during lethal endotoxemia plays a significant role in the long-term survival observed in animals pretreated with anti-N-PCT.

Table 1

DISCUSSION

This paper deals with a very important and relevant clinical issue, which is septic shock-induced morbidity and mortality. In contrast to the transiently increased of these proinflammatory 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 of 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-1 β 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 proinflammatory 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 confirm that the amino-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 of circulating early proinflammatory 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 for reinforcement and expanding the important role of N-PCT in septic shock.

The results of this study confirm that injection of a high dose of LPS (i.e., 15 mg/kg) 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. Here, we show that this septic-shock-like state induced by LPS correlates with disease severity and outcome, and was coupled to an early, progressive and sustained increase in plasma ProCT levels without corresponding CT secretion [14]. We also found an increased expression of Calc-1 gene and ProCT in the hypothalamus in response to lethal endotoxemia. In addition, we observed a very high induction in a set of mediators that has been implicated in inflammatory processes (i.e., IL-1 β , TNF- α and IL-10). However, and in contrast to ProCT, TNF- α and IL-1 β rise and decline early during endotoxemia. LPS also activates the hypothalamic-pituitary-adrenal axis, and it 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 release of TNF- α and IL-1 β , 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 experimental studies suggest 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 septic hamsters with peritonitis doubled their death rate. Conversely, immunoneutralization with ProCT-reactive antiserum increased the survival in septic hamsters and pigs [22, 33], even when the ProCT-reactive antiserum is administered after the animals became moribund [46]. In addition, only the injection of human ProCT to septic animals worsened the outcome, while in healthy animals the administration of similar doses of ProCT did not show 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 via which ProCT interferes with immunological functions is not elucidated. Present findings support the functional significance of ProCT in terms of immune response and indicate that its amino-terminal peptide plays a key immunoregulatory role in both early and later course of severe sepsis.

Because the massive increase of circulating ProCT that persisted until animals died after LPS challenge we analyzed the effects of passive immunoneutralization of endogenous N-PCT on lethality induced by an LD₁₀₀ of LPS. Our results show that anti-N-PCT increased survival in endotoxemic rats. Furthermore, anti-N-PCT delays the onset mortality in nonsurvivor animals and provides lasting protection. In addition to preventing the mortality when anti-N-PCT was given before LPS, anti-N-PCT also diminished the mortality when it was administered after the acute-phase cytokine responses have peaked and resolved. In this case, the reduction of 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 occur as proximal events in the course of sepsis. In both late and early therapy, the only animals to survive at the predetermined time of euthanasia were those which 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 began after the early, acute-phase response to endotoxin, the significant protection conveyed by anti-N-PCT suggests that it might target late-acting mediators of lethal systemic inflammation. These results support and extend the prior findings in septic pigs 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 proinflammatory 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 N-PCT₄₄₋₅₇ 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-1 β [39].

Of physiological relevance is the observation that neutralization of N-PCT down-regulates the expression of 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 [47]. It has been postulated that presence of microbial infection-specific response elements or of 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]. Here 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 coordinated autonomic and behavioral response to the LPS challenge. However,

these findings do not demonstrate that this particular expression of 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 show that icv administration of N-PCT causes sickness-type behaviors 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 [48]. 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 [49]. 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 homeostasis [50]. 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 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-mediated protection against lethal endotoxemia may be multiple: ranging from increasing production of anti-inflammatory mediators (e.g., IL-10 and ACTH) to inhibition of LPS-induced release of proinflammatory cytokines, such as TNF α and IL-1 β . Our results show that immunoneutralization of N-PCT is able to reduce the exacerbated humoral immune responses induced by an LD₁₀₀ dose of endotoxin in rats. In most animals in our study, the plasma levels of TNF- α and IL-1 β decreased toward normal levels within the first day in rats treated with anti-N-PCT antibodies, despite a persisting critical clinical condition. In contrast to TNF- α and IL-1 β , 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 leukocytes [20], present results 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 previously reported 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]. Here, we found that plasma levels of ACTH are maintained after anti-N-PCT during the first 24 h, suggesting that ACTH could play an important role in survival of endotoxemic animals treated with anti-N-PCT. Furthermore, other additional mechanisms may be involved in anti-N-PCT-mediated protection against lethal endotoxemia.

Present results also indicate that anti-N-PCT increases survival time in nonsurvivor rats and plays a direct role in long-term survival. These effects correlate with normalization in TNF- α and IL-1 β levels. An additional mechanism to explain the protective effect of anti-N-PCT in long-term survivors is the increase in the delayed production of counterregulatory anti-inflammatory mediators such as IL-10 and ACTH. In this context, the balance between TNF- α and IL-10 is important for immune homeostasis maintenance. Exuberant production of TNF- α contributes to an

overwhelming inflammatory response and tissue damage [51]. However, an increase in TNF- α is counterbalanced by simultaneous synthesis of an anti-inflammatory cytokine, IL-10, which suppresses production of many activating and regulatory mediators [52]. Here, we found that, in contrast to endotoxemic animals, all of which progressively worsened and finally died physiological parameters and IL-10 levels of anti-N-PCT-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 plus anti-N-PCT treatment still manifest symptoms. Interestingly, ACTH levels are significantly increased in long-term survivor animals treated with anti-N-PCT. These data suggest that the augmentation by anti-N-PCT of anti-inflammatory mediators' secretion most likely shifts the cytokine balance in the anti-inflammatory direction.

There are, however, potential limitations of the present study. First of all, although the animal model we used is comparable with the multiple organ failure that occurs in humans with preterminal 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. Second, 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-PCT₄₄₋₅₇) 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. Present data 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 for improving the unfavorable prognosis of septic shock.

In summary, we have shown that specific immunoneutralization of N-PCT can inhibit LPS-induced lethality in rats. The ability of anti-N-PCT antibodies to inhibit the production, release, or action of proinflammatory cytokines partially accounts for the protective effect of anti-N-PCT. 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, preliminary data in a model of sepsis induced by cecal 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 antibodies started after the induction of sepsis, at a time when rats show clear signs of sepsis, significantly improved survival. These experimental data provide strong support for the concept of N-PCT-targeted therapy for septic shock.

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FIGURE LEGENDS

Figure 1. Suppressive effect of anti-N-PCT₄₄₋₅₇ on N-PCT-induced anorexia. (A and B) Food intake was measured at 2, 4 and 24 h after a single icv injection either 1 nmol (5 µg/rat) N-PCT or control (aCSF/heat denaturated N-PCT, NRA/anti-NPCT) or anti-N-PCT (0.5, 1 or 2 µg/rat) 30 min before icv administration of 1 nmol N-PCT in adult male Wistar rats. Because there was no significant difference between control NRA (IgG) and anti-N-PCT, these data were integrated in the same bar. Data are expressed as means ± SEM (n= 8 animals per group). ***P < 0.001 vs. N-PCT-treated animals (ANOVA, Student-Newman-Keuls post hoc test).

Figure 2. Survival rates of rats challenged with LPS. Rats were given 1, 5, 10 or 15 mg/kg of LPS by ip administration. The data 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 daily for at least 7 days. There were 10 animals in each group. The survival rate was estimated by the Kaplan-Meier method and compared by long-rank test. **P < 0.01; and ***P < 0.001 vs. untreated rats.

Figure 3. Effects of anti-N-PCT on LPS-induced mortality and hypothermia. (A and B) Rats were injected ip with an LD₁₀₀ dose of LPS (15 mg/kg) at t=0 and were pretreated ip with PBS, NRA, or 50, 100 and 200 µg/kg anti-N-PCT₄₄₋₅₇, or 100 µg/kg monoclonal anti-N-PCT 1 h earlier (n=12 for each group). Rat mortality was monitored twice daily for at least 7 days. The survival rate was estimated by the Kaplan-Meier method and compared by long-rank test. **P < 0.01; and ***P < 0.001 vs. control anti-N-PCT (NRA)+ PBS-treated rats. Body temperature data are mean ± SEM. **P < 0.01; and ***P < 0.001 vs. control NRA + LPS-treated animals (ANOVA, Student-Newman-Keuls post hoc test). Because there was no significant difference between NRA + PBS or anti-N-PCT + PBS, these data were integrated in the same curve.

Figure 4. Effect of prophylactic and therapeutic anti-N-PCT administration on survival after a lethal dose of LPS. Rats (n=12 in each group) received an LD₁₀₀ of LPS (15 mg/kg, ip). Anti-N-PCT₄₄₋₅₇ (200 µg/kg, ip) was administered either 1 h before (anti-N-PCT/LPS) or 2 h after (LPS/anti-N-PCT) a LD₁₀₀ dose of LPS (15 mg/kg). (A) The data 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. Delayed administration of anti-N-PCT still conferred significant protection against LPS-induced lethality. Rat mortality was monitored twice daily for at least 7 days. In a parallel set of endotoxemic rats, survival was monitored for up 3 wk. The Kaplan-Meier method was used to compare the differences in mortality rates between groups. **P < 0.01; and ***P < 0.001 vs. LPS-treated animals. In (B), the average survival time was calculated for nonsurvivor in both the untreated and anti-N-PCT groups. ***P < 0.001 vs. LPS-treated animals; †P < 0.01 vs. LPS + anti-N-PCT-treated animals (ANOVA, Student-Newman-Keuls post hoc test).

Figure 5. Anti-N-PCT pre-treatment reduces LPS-induced hypothalamic Calc-1 gene expression and ProCT production in plasma and hypothalamus. Rats were injected with a single dose of anti-N-PCT₄₄₋₅₇ (200 µg/kg, ip) as indicated, followed 1 h later by an LD₁₀₀ dose of endotoxin (LPS, 15 mg/kg, ip). Plasma and protein extract from hypothalamus were collected at different time points after endotoxin injection; and the Calc-1 gene, ProCT and CT contents were determined as described in Material and Methods. Because there was no significant difference between NRA + PBS and anti-N-PCT + PBS, these data were integrated in the same curve. Data are presented as means ± SEM (n= 8 rats at 1, 3 and 6 h; n= 7 at 12 h; n= 6 at 24 h). **P < 0.01; and ***P < 0.001 vs. NRA + LPS-challenged animals (ANOVA, Student-Newman-Keuls post hoc test).

Figure 6. Effects of pre-treatment with anti-N-PCT on LPS-induced release of TNF-α, IL-1β, IL-10 and ACTH in plasma. Rats were injected ip with an LD₁₀₀ dose of LPS (15 mg/kg). Anti-N-PCT₄₄₋₅₇ (200 µg/kg, ip) 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 sacrificed 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 data were integrated in the same curve. Data are presented as means ± SEM (n= 8 rats at 1, 3 and 6 h; n= 7 at 12 h; n= 6 at 24 h). *P < 0.05; **P < 0.01; and ***P < 0.001 vs. NRA + LPS-treated animals (ANOVA, Student-Newman-Keuls post hoc test).

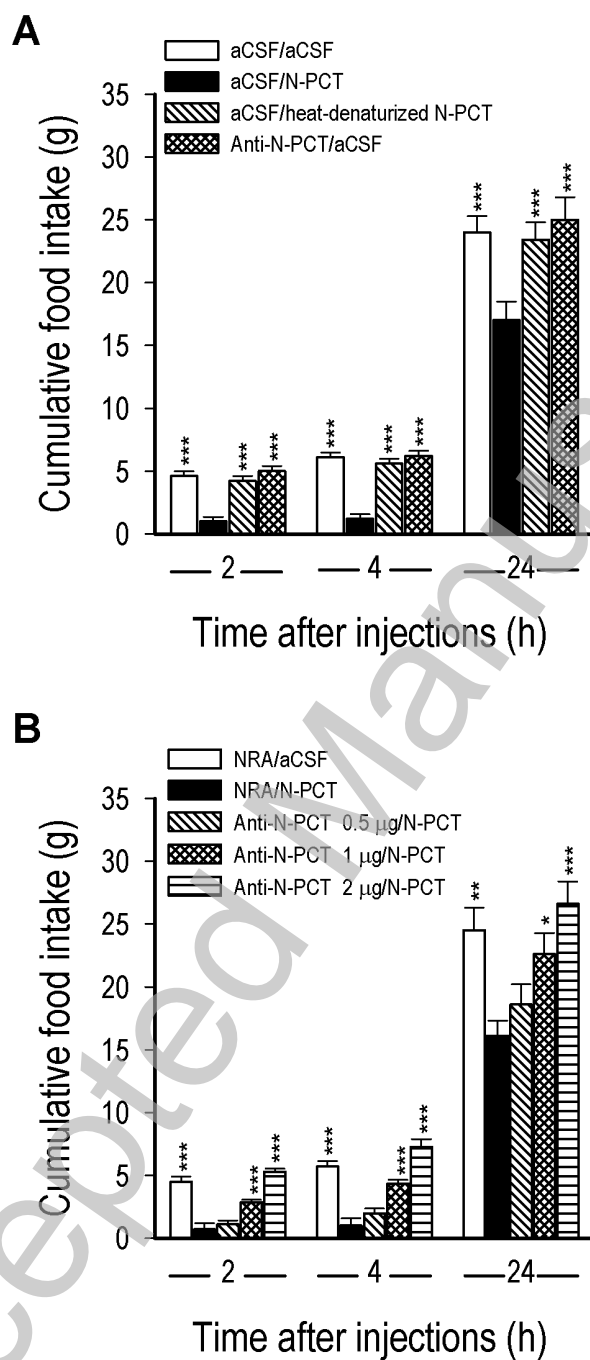


Figure 1

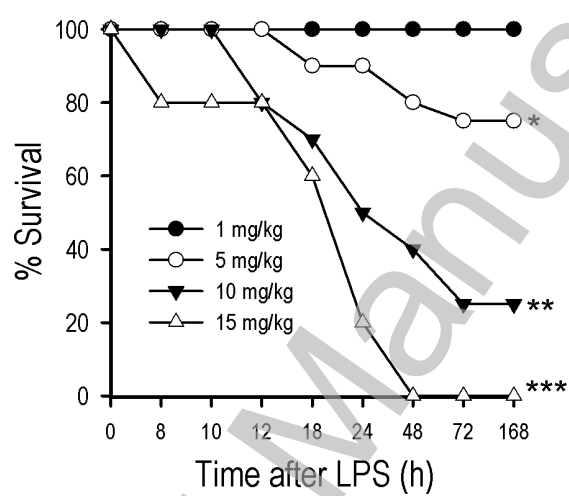


Figure 2

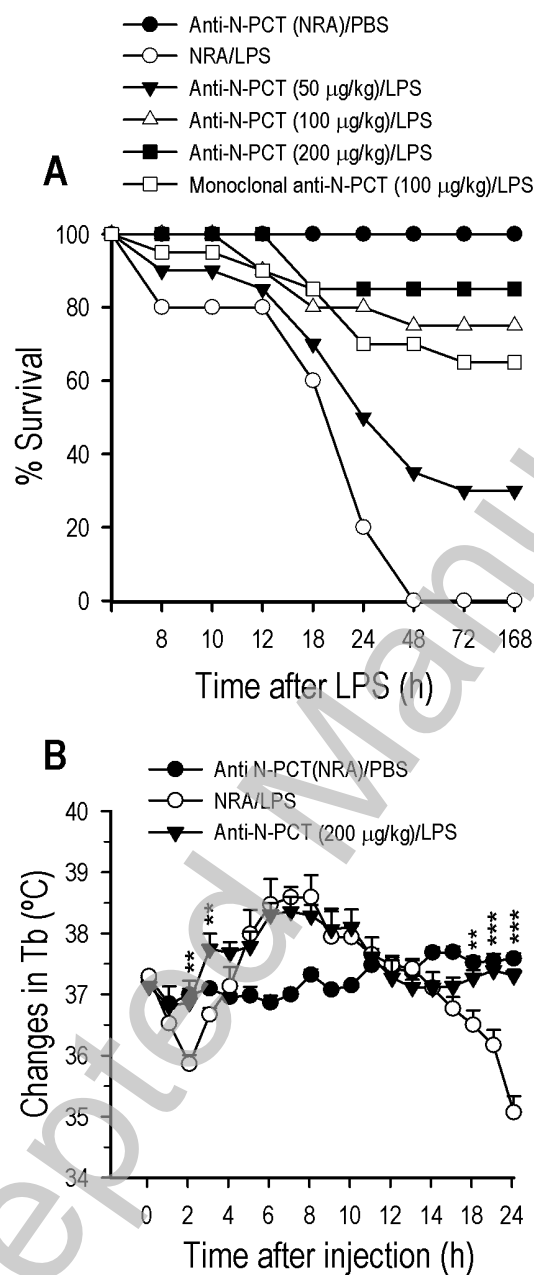


Figure 3

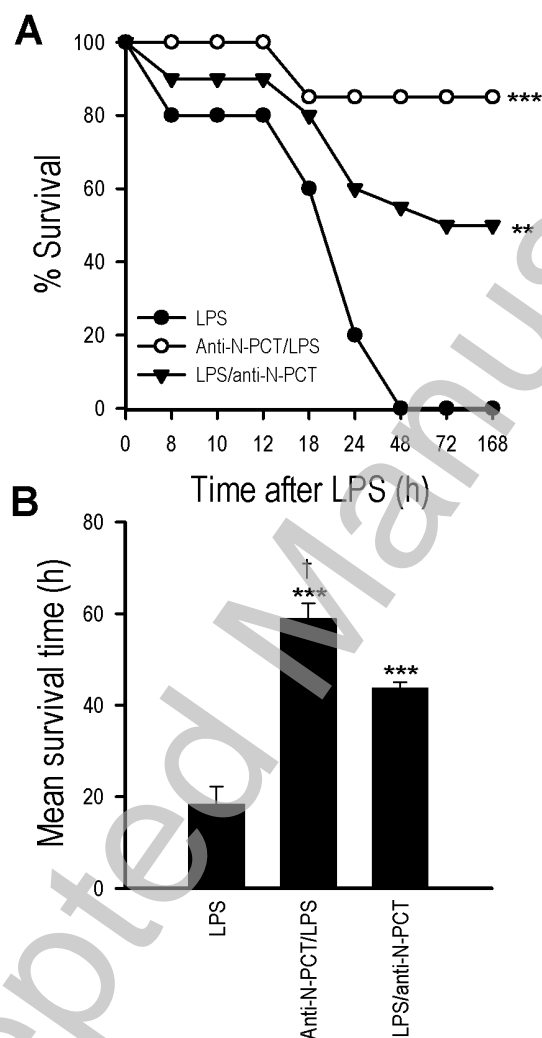


Figure 4

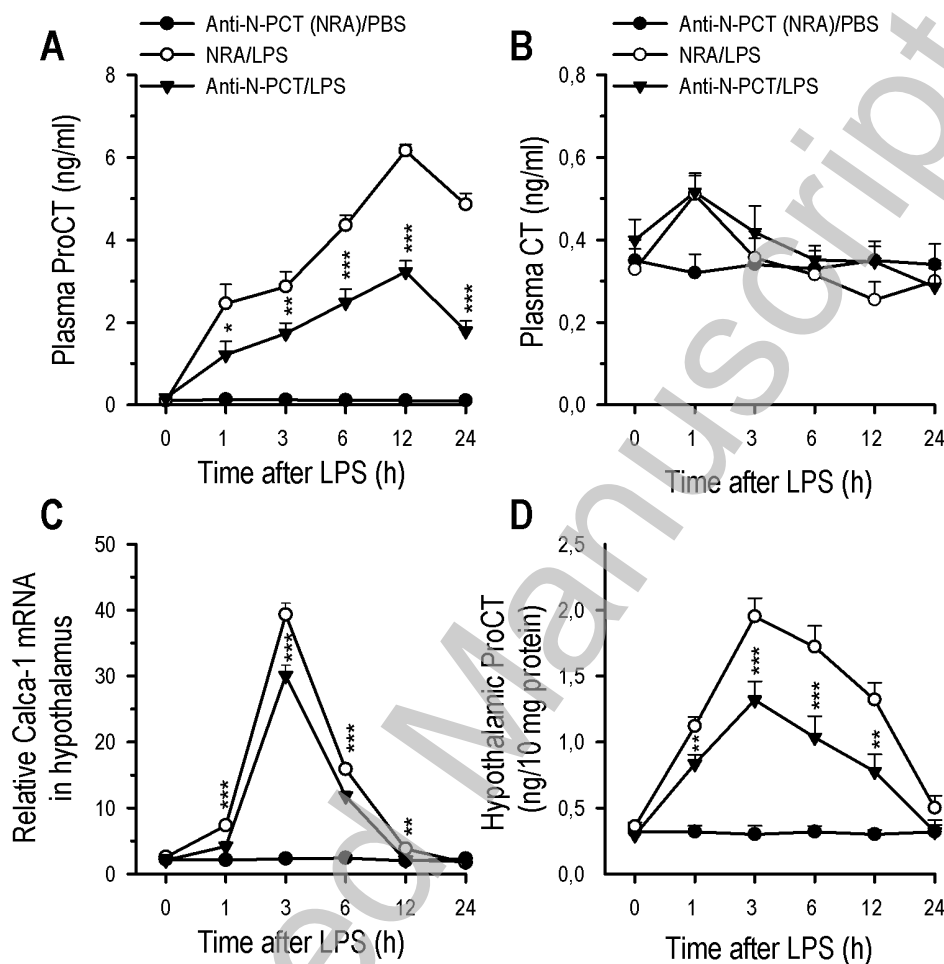


Figure 5

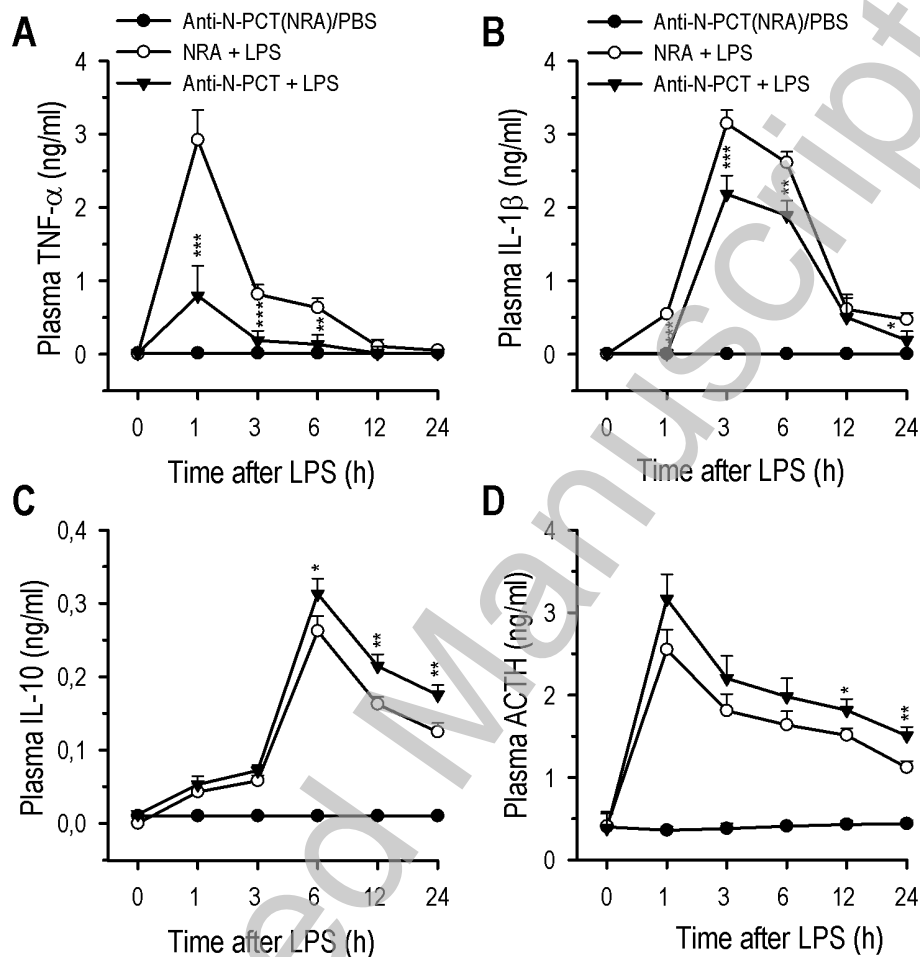


Figure 6

Table 1. Effect of anti-N-PCT on plasma cytokine, ACTH, CT and ProCT levels (ng/ml) 3 and 7 d after LPS administration in long-term surviving animals

Mediator	Time [h] after LPS-treatment	Anti-N-PCT + PBS	Anti-N-PCT + LPS
TNF- α	0	≤ 0.015	≤ 0.015
	72	≤ 0.015	≤ 0.015
	168	≤ 0.015	≤ 0.015
IL-1 β	0	≤ 0.005	≤ 0.005
	72	≤ 0.005	0.08 ± 0.02
	168	≤ 0.005	≤ 0.005
IL-10	0	≤ 0.010	≤ 0.010
	72	≤ 0.010	$0.11 \pm 0.02^*$
	168	≤ 0.010	$0.07 \pm 0.02^*$
ACTH	0	0.40 ± 0.12	0.38 ± 0.10
	72	0.57 ± 0.12	$1.68 \pm 0.40^*$
	168	0.48 ± 0.10	$0.77 \pm 0.21^*$
CT	0	0.39 ± 0.12	0.37 ± 0.10
	72	0.41 ± 0.10	0.46 ± 0.16
	168	0.43 ± 0.18	0.41 ± 0.12
ProCT	0	≤ 0.10	≤ 0.10
	72	≤ 0.10	0.17 ± 0.10
	168	≤ 0.10	0.13 ± 0.05

Animals were pre-treated ip with either 200 μ g/kg of control NRA (IgG) or anti-N-PCT₄₄₋₅₇ 1 h before an LD₁₀₀ dose of LPS (15 mg/kg). Data are presented as mean \pm SEM of 4 rats per group. The groups of animals treated with NRA + LPS or PBS + LPS have not been included because no animals survived at these point times. * $P < 0.05$ versus control basal levels (time 0).