Inhibitory Effects of the Peptide (CKPV)\textsubscript{2} on Endotoxin-Induced Host Reactions

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Background. α-Melanocyte stimulating hormone (α-MSH) is an endogenous peptide that has remarkable anti-inflammatory and antimicrobial effects. These activities have been traced to the C-terminal tripeptide Lys-Pro-Val (KPV). A dimer composed of two KPV sequences connected with a Cys-Cys linker, (CKPV)\textsubscript{2}, is currently under clinical investigation for antimicrobial use. The present research was designed to evaluate effects of (CKPV)\textsubscript{2} on endotoxin-induced host reactions in vitro and in vivo.

Materials and methods. Effects of (CKPV)\textsubscript{2}, KPV, and [Nle\textsubscript{4}-dPhe\textsubscript{7}]-α-MSH (NDP-α-MSH) on tumor necrosis factor α (TNF-α) production were determined: 1) in human peripheral blood mononuclear cells (PBMC) stimulated with lipopolysaccharide (LPS) in vitro, and 2) in rats injected with LPS i.v. and sacrificed at 1 h. In additional experiments, dialysis peritonitis was induced in rats by adding LPS to dialysis fluid. Net ultrafiltrate was calculated and concentrations of nitrite (NO\textsubscript{2}) and TNF-α were measured in blood and peritoneal fluid at 7 h.

Results. (CKPV)\textsubscript{2} inhibited TNF-α production by LPS-stimulated human PBMC. This small peptide was as effective as NDP-α-MSH and more potent than KPV. Similar effectiveness was observed in vivo: 1 h after LPS injection, the large increase in circulating TNF-α was markedly reduced by (CKPV)\textsubscript{2} treatment. In LPS-induced peritonitis, (CKPV)\textsubscript{2} restored net ultrafiltrate to control values and significantly inhibited concentrations of TNF-α and NO\textsubscript{2} both in plasma and in dialysate.

Conclusions. The remarkable capacity of (CKPV)\textsubscript{2} to inhibit endotoxin-induced host reactions suggests that it may be useful in treatment of inflammatory disorders.© 2005 Elsevier Inc. All rights reserved.

Key Words: α-Melanocyte stimulating hormone (α-MSH); synthetic melanocortins; peritoneal dialysis; endotoxin; peritonitis; nitric oxide (NO); tumor necrosis factor α (TNF-α).

INTRODUCTION

α-Melanocyte stimulating hormone (α-MSH) is an endogenous tridecapeptide that exerts immunomodulatory, antipyretic, and anti-inflammatory effects [1–4]. Further, recent data indicate that α-MSH and related melanocortins have antimicrobial effects [5]. Pre-clinical studies demonstrated that treatment with α-MSH successfully inhibited inflammation in models of acute [6] and chronic inflammatory disorders including adjuvant arthritis [7] and inflammatory bowel disease [8]. Treatment with α-MSH improved survival in mice with septic shock induced by cecal ligation and puncture [9] and greatly reduced neutrophil migration into the lungs of rats with acute respiratory distress syndrome (ARDS) caused by intratracheal injection of endotoxin [9]. Therefore, disorders characterized by acute, chronic, or systemic inflammation might respond to treatment with α-MSH peptides. Concurrent antimicrobial properties can make melanocortins particularly promising as therapeutic agents when inflammation and infection occur together [10].

α-MSH peptides are, therefore, a potential new class of drugs, provided that cost-effective and stable analogs can be developed [11]. In pursuit of such analogs, we focused on the C-terminal sequence α-MSH (11–13).
This tripeptide, composed of Lys-Pro-Val (KPV), exerts anti-inflammatory and antimicrobial influences that, although less potent on a molar basis, are similar to those of the parent α-MSH (1–13) molecule [2, 5, 12, 13]. A dimer obtained by inserting a Cys-Cys linker between two units of KPV, (CKPV)2 (Fig. 1) showed remarkable candidacidal effects [14] and very low toxicity (J.M. Lipton, unpublished observations). Therefore, we extended the research to determine whether this molecule also has anti-inflammatory properties much as α-MSH (1–13) and (11–13). To this purpose, we determined effects of (CKPV)2 on production of the inflammatory cytokine TNF-α by LPS-stimulated human PBMC. Potency of (CKPV)2 was compared to those of KPV and of the potent α-MSH analogue [Nle4-dPhe7]-α-MSH (NDP-α-MSH) [15]. Because (CKPV)2 was as effective as NDP-α-MSH and more potent than the monomer KPV the peptide was subjected to further investigations to determine the capacity of (CKPV)2 to antagonize endotoxin-induced host reactions in vivo. The anti-TNF-α properties of (CKPV)2 were investigated after intravenous LPS injection in rats. Further, in a rat model of LPS-induced peritonitis during peritoneal dialysis, we determined effects of (CKPV)2 on changes in net ultrafiltrate and inflammatory mediator production. Peritoneal dialysis is an established therapy for end-stage renal failure and survival of patients under this regimen is equivalent to that under hemodialysis. Peritonitis is a significant cause of morbidity that often leads to change of treatment to hemodialysis [16] and, therefore, control of this complication is very important. Repeated attacks of peritonitis damage the peritoneal membranes causing permeability changes and are the main reasons for technique failure [16]. Cytokines and nitric oxide (NO) are significant tissue-damaging factors induced during the inflammatory reaction [17, 18]. Toxic shock syndrome can also occur as a severe complication of peritoneal dialysis [19]. Therefore, we elected to test the anti-inflammatory properties of (CKPV)2 in a model of dialysis peritonitis. This experimental model allows accurate evaluation of the effects of treatment on local and systemic inflammatory mediators. Consequently, the results provide a general index of the anti-inflammatory potential of treatment.

METHODS

LPS-Stimulated Human PBMC

Heparinized blood (30 ml) was obtained from seven normal subjects. Peripheral blood mononuclear cells (PBMC) were isolated from blood by density centrifugation through Ficoll-Hypaque (Sigma Chemical Co., St. Louis, MO). Cells were washed twice in sterile PBS and suspended in RPMI 1640 (Life Technologies, Inc., Paisley, United Kingdom) supplemented with 10 mM HEPES (Sigma) 2 mMglutamine (Gibco), 10% FCS (Gibco), 50 U/ml penicillin (ICN Flow, Costa Mesa, CA), and 50 μg/ml streptomycin (ICN Flow). PBMC were seeded in 24 well flat-bottomed plates at the density of 2.5 × 10⁵ cells per well, and incubated in 5% CO₂ atmosphere at 37°C for 24 h in presence of: 1) medium alone; 2) medium plus (CKPV)2 10⁻⁶ to 10⁻⁴ M; 3) medium plus LPS from Escherichia coli 055:B5 (Sigma Chemical Co.), 1 ng/ml; 4) medium plus LPS 1 ng/ml plus KP, (CKPV)2 or [Nle4-dPhe7]-α-MSH all 10⁻⁶ to 10⁻⁴ M. After a 24 h-incubation, samples were centrifuged and supernatants separated and stored at −80°C. The experiments were run in triplicate. All of the synthetic melanocortins were kindly provided by Prof. Paolo Grieco, Università Federico II, and Napoli, Italy.

Acute Endotoxemia

Male Wistar rats, 200 to 220 g (Charles River, Calco, Italy) were maintained at the animal care facilities of Ospedale Maggiore di Milano. The animals received care in compliance with the Principles of Laboratory Animal Care, formulated by the National Society of Medical Research, and the Guide for the Care and Use of Laboratory Animals, prepared by the National Academy of Sciences, and published by the National Institutes of Health (NIH Publication No. 85-23, revised 1985).

Rats (N = 6 per group) received i.v. bolus injections of LPS (50 μg in 200 μl) along with either 1) saline (200 μl); 2) KP 55 μg; 3) (CKPV)2 140 μg; or 4) NDP-α-MSH 237 μg. Peptide doses were calculated to be equimolar and dissolved in 200 μl saline. Rats were sacrificed 1 h after treatment under ether anesthesia. Blood was obtained from the inferior vena cava.

LPS-Induced Peritonitis

Five groups of rats (n = 8 per group) received under light ether anesthesia intraperitoneal injections of either: 1) 15 ml dialysis fluid (CAPD/DPCA3, 4.25% glucose, Fresenius Medical Care, Bad Homburg, Germany); or 2) dialysis fluid plus (CKPV)2 10⁻⁵ M (final concentration); or 3) dialysis fluid plus LPS 5 μg/ml; 4) dialysis fluid plus LPS and (CKPV)1 10⁻⁵ M; or 5) dialysis fluid plus LPS and 140 μg (CKPV)2 given as an iv bolus. After 7 h, each rat was sacrificed under ether anesthesia and the abdomen incised. Peritoneal fluid was removed, measured, centrifuged, aliquoted, and stored at −80°C. Blood was obtained from the inferior vena cava.

Assays

Human and rat TNF-α was measured using specific commercial immunoassay kits (Biosource International, Camarillo, CA). Plasma nitrite concentration in rats was determined as a measure of NO release. Nitrites (NO₂⁻) were converted into nitrates (NO₃⁻) by treatment of serum with nitrate reductase (Boehringer Mannheim Italia SpA, Milan, Italy). After enzymatic reduction, samples were mixed with equal amounts of Griess reagent (sulfanilamide 1%, naphthylethylenediamine 0.1% in phosphoric acid 0.25%). Samples were incubated at room temperature for 10 min and absorbency was measured at 540 nm using a automated microplate reader. The effect of (CKPV)2 on peritoneal transport of small and large molecules was evaluated by measuring dyesulfate/sulfate ratio of glucose, urea, creatinine, and total proteins using specific kits (Roche Diagnostics, Mannheim, Germany) on a modular autoanalyzer.
Statistical Analysis

Data were analyzed using one-way ANOVA procedures followed by Dunnett’s test for multiple comparisons of group means. Two-tail probability less than 0.05 were considered significant. Data are expressed as mean ± SE.

RESULTS

Human PBMC

PBMC incubated with medium alone or concentrations of (CKPV)₂ did not release significant TNF-α (not shown). However, upon LPS stimulation, PBMC produced and released substantial amounts of TNF-α. When LPS-stimulated cells were coincubated with concentrations of (CKPV)₂ there was a significant dose-related inhibition of TNF-α release (P < 0.05 for all concentrations versus control). The magnitude of the (CKPV)₂ effect was similar to that of equimolar NDP-α-MSH (P > 0.05 for all concentrations) and greater than that of KPV at 10 and 100 μM concentrations (P < 0.05). Data are expressed as percent response (mean ± SE) relative to TNF-α production by LPS-stimulated PBMC incubated with medium alone.

LPS-Induced Circulating TNF-α

Circulating TNF-α measured 1 h after LPS injection was greatly increased above control concentrations (Fig. 3). Administration of the melanocortin peptides (KPV), (CKPV)₂, or NDP-α-MSH markedly inhibited these increases (Fig. 3). Similar to in vitro results, (CKPV)₂ potency was comparable to that of NDP-α-MSH. (CKPV)₂ was superior to the monomer KPV in inhibiting the LPS-induced TNF-α response. At this short 1 h interval there was no increase in plasma NO₂⁻ concentration.

LPS-Induced Peritonitis

Addition of LPS to dialysis fluid caused a marked reduction in net ultrafiltrate measured at 7 h (Fig. 4). (CKPV)₂, given either i.p. or i.v. at the same dose, effectively prevented this effect of LPS (Fig. 4). The peptide did not alter appreciably the peritoneal transport of small and large molecules as determined by measures of dialysate/serum ratio of glucose, urea, creatinine, and total proteins (not shown).

LPS-induced peritonitis was marked by a substantial increase in NO₂⁻ concentrations in plasma and dialysate. Treatment with (CKPV)₂, given i.p. or as an i.v. bolus at the same dose, significantly inhibited circulating and peritoneal NO₂⁻ concentrations (Fig. 5).


(CKPV)₂ given in the absence of LPS, did not alter NO₂⁻ concentrations in plasma or peritoneal fluid.

Plasma TNF-α measured at the 7 h interval, was still above control levels in LPS-treated animals, but concentrations were declining relative to samples obtained at 1 h. (CKPV)₂ inhibited TNF-α concentrations in plasma and dialysate of endotoxin-treated rats (Fig. 6 A, B), but at this late interval the magnitude of the effect was less prominent compared to 1 h, when TNF-α concentrations were much greater.

**DISCUSSION**

The present results indicate that the synthetic peptide (CKPV)₂ antagonizes inflammatory reactions elicited by endotoxin in vitro and in vivo. This small and relatively inexpensive peptide inhibited TNF-α production as effectively as the super potent α-MSH analogue NDP-α-MSH. Further, the peptide reduced local and systemic host reactions during LPS-induced dialysis peritonitis in rats.

The conception that α-MSH and related melanocortins exert their effects through binding to specific melanocortin receptors (MCRs) [11]. Recognition and cloning of MCRs has greatly improved understanding of peptide-target cell interactions [26]. Synthetic melanocortins with selective affinities for individual MCR may soon form the basis for new classes of therapeutic molecules [11]. The five MCRs cloned thus far belong to the class A of guanine nucleotide-binding protein (G-protein)-coupled, seven transmembrane receptors. All are functionally coupled to adenyl cyclase and mediate their effects primarily by activating a cyclic 3',5'-adenosine monophosphate (cAMP)-dependent signaling pathway. It appears that several MCR subtypes contribute to the anti-inflammatory effects of melanocortin peptides, perhaps in different physiological or pathological conditions, in different tissues or at different peptide concentrations.

The peptide KPV, which corresponds to the α-MSH (11–13), is considered the anti-inflammatory “message sequence” of α-MSH. Although this tripeptide is not as...
potent as the full-length α-MSH (1–13) amino acid sequence, it reduces fever, inflammation, and inflammatory mediator production in several pre-clinical studies [5, 12, 13, 27–38]. Further, it exerts similar inhibitory effects on NF-κB activation [39–41]. However, several observations indicate that this molecule does not compete with α-MSH for receptors expressed by the B16 mouse melanoma cells [42] and does not recognize any of the known melanocortin receptors [25, 26, 38, 43]. Therefore, the cell receptor for Lys-Pro-Val is still unknown.

KPV has been used as a template to design peptidomimetics [44]. In a search for novel peptides based on KPV, we designed the dimer (CKPV)2 composed of two KPV units connected with a Cys-Cys linker [14]. The three-dimensional structure of the dimer was determined using NMR spectroscopy. The conformational study indicated that (CKPV)2 is characterized by an extended backbone structure and showed that the peptide adopts a β-turn-like structure at Pro3-Val-NH2 4. Of interest, this structure reproduces the conformation of α-MSH (1–13) [45]. It is likely that this folded structure helps interaction with melanocortin receptors and may explain the greater potency of the dimer relative to the KPV monomer.

Peritonitis is a common complication of peritoneal dialysis. Dialytic peritonitis is caused by microbial invasion, which induces release of cytokines and other mediators responsible for local and systemic inflammation. Immune cells such as monocytes, lymphocytes, neutrophils, and mast cells emigrate from vessels into the peritoneal cavity in response to microbial invasion and start local immune reactions [46]. Although such reactions contribute to eradicate infection, repeated episodes of local inflammation can damage the peritoneal membrane. Medications that attenuate local and systemic host reactions can, therefore, prolong duration of peritoneal dialysis and reduce patient discomfort. The most common functional alteration during peritoneal dialysis is increased rate of peritoneal small-solute transport, resulting in impaired ultrafiltration and decreased dialysis efficiency [47]. Ultrafiltration failure that accompanies chronic peritoneal dialysis is believed to be caused by molecules such as the water channel aquaporin-1 and NO, which regulate effective peritoneal surface area and microvascular permeability [48]. NO-mediated increase in peritoneal surface area, followed by a dissipation of the osmotic gradient, contributes to the loss of ultrafiltration. Addition of NO inhibitors to the dialysate significantly improved ultrafiltrate and reversed permeability modifications in rats with peritonitis [49, 50] much as (CKPV)2 in the present research.

(CKPV)2 is presently under investigation in a phase IIb clinical trial for its antimicrobial effects. The remarkable capacity of the peptide to inhibit endotoxin-induced host reactions shown in the current research suggests that it may also be useful in the treatment of inflammatory disorders.

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