Design and Synthesis of Melanocortin Peptides with Candidacidal and Anti-TNF-α Properties

ARTICLE *in* JOURNAL OF MEDICINAL CHEMISTRY · APRIL 2005

Impact Factor: 5.45 · DOI: 10.1021/jm040890j · Source: PubMed

Design and Synthesis of Melanocortin Peptides with Candidacidal and Anti-TNF-α Properties

Paolo Grieco,[†] Claudia Rossi,[‡] Stefano Gatti,[§] Gualtiero Colombo,[‡] Andrea Carlin,[‡] Ettore Novellino,[†] Teresa Lama,[†] James M. Lipton,[∥] and Anna Catania[‡,*]

Department of Pharmaceutical Chemistry and Toxicology, Università di Napoli, 80131 Napoli, Italy; Divisions of Internal Medicine and Liver Transplantation, Ospedale Maggiore di Milano IRCCS, 20122 Milano, Italy; and Zengen Inc., Woodland Hills, California 91367

Received October 20, 2004

α-Melanocyte stimulating hormone (α-MSH) is an endogenous antiinflammatory peptide with antimicrobial properties. We recently found that a synthetic analogue, [DNal(2′)-7, Phe-12]-α-MSH (6−13), was considerably more potent in killing *Candida albicans,* but the anti-cytokine potential of the molecule was not investigated. Because molecules that combine candidacidal and antiinflammatory properties could be very useful in clinical practice, we measured the anti-TNF-α potential of [DNal(2′)-7, Phe-12]-α-MSH (6−13) and explored effects of amino acid deletions and substitutions on both anti-*Candida* and anti-TNF-α activities. The results show that anti-TNF-α properties of this candidacidal peptide are only marginally increased relative to the native sequence. Conversely, we found that a closely related candidacidal analogue, [DNal(2′)-7, Pro-12]-α-MSH (6−13), had enhanced anti-TNF-α effects in vitro and in vivo. This peptide, and other melanocortins with a similar dual effect, could be very useful to eradicate infections and, concurrently, reduce inflammatory reactions.

Introduction

Adrenocorticotropic hormone (ACTH) and α-, *β*-, and *γ*-melanocyte stimulating (α-, *β*-, *γ*-MSH) hormones derive from posttranslational processing of the precursor molecule proopiomelanocortin (POMC)[1] and are collectively called melanocortin peptides or melanocortins. α-MSH is a tridecapeptide that exerts pleiotropic effects in several physiological pathways including modulation of fever and inflammation,[2−4] control of feeding behavior and energy homeostasis,[5,6] control of autonomic functions,[7−9] and increase in melanogenesis.[10−12] Recent research indicates that α-MSH also has broad antimicrobial influences.[13,14] α-MSH inhibits growth of both the yeast *Candida albicans* and the Gram-positive bacterium *Staphylococcus aureus.*[13] Antimicrobial effects of α-MSH occur through increases in cAMP, with remarkable similarity to the signaling pathway in mammalian cells in which α-MSH binds to melanocortin receptors and exerts its influence primarily by activating a cAMP-dependent pathway.[15−17]

In a search for synthetic α-MSH analogues with superior antimicrobial properties over the natural molecule, we recently designed and synthesized novel peptides based on the α-MSH (6−13) sequence H-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2.[18] Several of these molecules had greater candidacidal activity relative to the nonsubstituted fragment. The most potent of these compounds was [DNal(2′)-7, Phe-12]-α-MSH (6−13), that killed almost 100% of yeast cells in repeated experiments. The candidacidal potency of this peptide was also superior to that of the complete α-MSH (1−13) sequence. To better characterize the structure−activity relation of [DNal(2′)-7, Phe-12]-α-MSH (6−13), the present research explored influences of amino acid deletions and substitutions on its candidacidal activity.

Because the native α−MSH (1−13) molecule and certain of its derivatives exert anti-cytokine influences,[3,19−22] we designed experiments to determine whether [DNal(2′)-7, Phe-12]-α-MSH (6−13) and related amino acid sequences also have both candidacidal and anti-cytokine influences. To this purpose, we performed parallel experiments that determined the capacity of each peptide to inhibit release of the proinflammatory cytokine TNF-α by human cells and to kill *C. albicans*. Although [DNal(2′)-7, Phe-12]-α-MSH (6−13) did not possess enhanced anti-TNF-α properties, the related candidacidal peptide [DNal(2′)-7, Pro-12]-α-MSH (6−13) did have remarkable anti-TNF-α activity. Therefore, we explored anti-TNF-α influences of the molecule in a model of endotoxemia in vivo.

Results

Candidacidal and Anti-TNF-α Properties of Synthetic Melanocortins. The α-MSH analogue [DNal(2′)-7, Phe-12]-α-MSH (6−13) (peptide **1**) inhibited *C. albicans* colony formation almost completely (Table 1, Figure 1). However, the capacity of this potent candidacidal peptide to inhibit TNF-α release by endotoxin-stimulated human PBMC was not enhanced compared with the nonsubstituted peptide α-MSH (6−13) bearing phenylalanine in position 7 and proline in position 12 (Figure 1). Therefore, we attempted to identify the individual influences of DNal(2′)-7 and Phe-12 substitu-

* Corresponding author: Anna Catania, M. D., Divisione di Medicina Interna 1, Padiglione Granelli, Ospedale Maggiore di Milano IRCCS, Via F. Sforza 35, 20122 Milano, Italy. E-mail anna.catania@unimi.it. Tel/FAX +39 02 5503 3318.

[†] Università di Napoli.

[‡] Division of Internal Medicine, Ospedale Maggiore di Milano IRCCS.

[§] Division of Liver Transplantation, Ospedale Maggiore di Milano IRCCS.

[∥] Zengen Inc.

10.1021/jm040890j CCC: $30.25
Published on Web 02/02/2005

Table 1. Effect of α-MSH (6−13) Derivatives (10^{-4} M) on *C. albicans* CFU and TNF-α Release by Endotoxin-Stimulated Human PBMC

peptide no.	amino acid sequence	*C. albicans*, % inib[a]	SD	TNF-α, % inhib[a]	SD
α-MSH$_{6-13}$	H-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	59.4	15.5	12.3	4.1
1	H-His-**dNal(2′)**-Arg-Trp-Gly-Lys-**Phe**-Val-NH_2	99.8	0.4	18.7	6.7
2	H-His-Phe-Arg-Trp-Gly-Lys-**Phe**-Val-NH_2	90.8	9.3	<10	
3	H-His-**D-Nal(2′)**-Arg-Trp-Gly-Lys-Pro-Val-NH_2	95.3	7.7	41.8	5.2
4	H-**D-Nal(2′)**-Arg-Trp-Gly-Lys-**Phe**-Val-NH_2	51.4	9.5	<10	
5	H−Arg-Trp-Gly-Lys-**Phe**-Val-NH_2	19.3	5.6	<10	
6	H-Trp-Gly-Lys-**Phe**-Val-NH_2	5.7	2.6	<10	
7	H-Gly-Lys-**Phe**-Val-NH_2	5.0	2.6	<10	
8	H-Lys-**Phe**-Val-NH_2	2.7	1.4	<10	
9	H-His-**dNal(2′)**-Arg-Trp-NH_2	43.0	13.4	13.9	4.8
10	H-**Ala**-**dNal(2′)**-Arg-Trp-Gly-Lys-**Phe**-Val-NH_2	62.8	9.8	<10	
11	H-His-**Ala**-Arg-Trp-Gly-Lys-**Phe**-Val-NH_2	49.9	8.7	<10	
12	H-His-**dNal(2′)**-**Ala**-Trp-Gly-Lys-**Phe**-Val-NH_2	95.7	1.8	23.0	9.7
13	H-His-**dNal(2′)**-Arg-**Ala**-Gly-Lys-**Phe**-Val-NH_2	24.9	7.9	18.1	4.1

[a] Versus control.

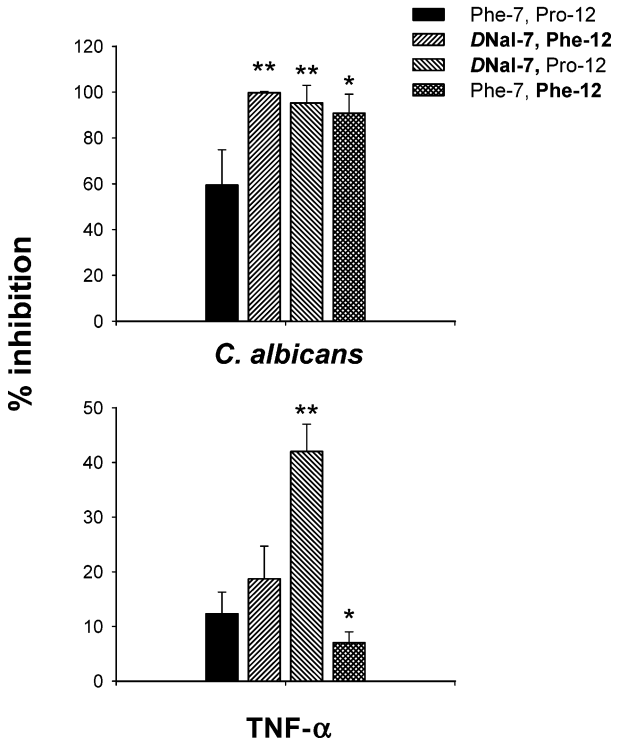

Figure 1. Candidacidal and anti-TNF-α effects of α-MSH-(6−13)-derived peptides. Inhibition of *C. albicans* viability (six strains) was evaluated as percent reduction in CFU in the presence of each peptide (upper panel) relative to control samples. Anti-TNF-α effects are shown as percent inhibition compared to saline control (lower panel). TNF-α release was measured in supernatants of endotoxin-stimulated human PBMC (five subjects) incubated with each peptide or control saline. Amino acid substitutions in the natural α-MSH (6−13) sequence (Phe-7, Pro-12) had distinctive influences on the candidacidal or anti-TNF properties of the peptide. When the natural Phe-7 residue was substituted with D-Nal(2′) both the candidacidal and the anti-TNF-α effects were greatly enhanced. Conversely, substitution of Pro-12 with Phe promoted *Candida* killing, but reduced the anti-TNF-α activity. Bars denote mean ± SD * $p < 0.05$ ** $p < 0.01$ vs non substituted α-MSH (6−13).

tions on candidacidal and anti-TNF-α effects. Results indicated that replacement of Phe-7 with D-Nal(2′) (peptide **2**) increased both the candidacidal and the anti-TNF-α effects of the α-MSH (6−13) sequence. Indeed, peptide **2** showed significantly greater candidacidal and anti-TNF-α activity than α-MSH (6−13). The mean

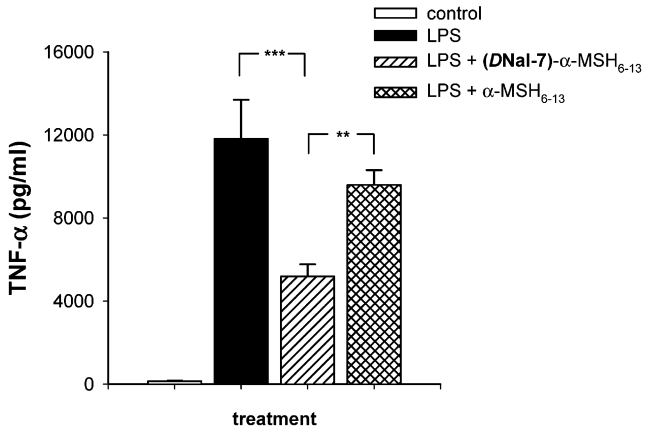

Figure 2. Influences of [D-Nal(2′)-7]-α-MSH (6−13) (peptide **2**) and native α-MSH (6−13) (500 μg/Kg iv) on plasma TNF-α concentrations in rats given acute LPS injection (250 μg/Kg iv). Rats ($N = 5$ per group) were sacrificed at 2 h. Bars denote mean ± SD. ** $p < 0.01$; *** $p < 0.001$.

inhibition of TNF-α by peptide **2** was 41.8% ± 5.2 SD vs 12.3% ± 4.1 for the nonsubstituted peptide. The inhibitory effect of peptide **2** on TNF-α production was also superior to that of the complete α-MSH (1−13) peptide that caused 28.6% ± 6.8 reduction in TNF-α release in the same experimental conditions ($p < 0.05$). An interesting observation was that, although peptide **2** was a very potent candidacidal peptide (mean inhibition of CFU was 95.3%), its activity was not as consistent as that of peptide **1**; the variability in killing (SD) was 7.7 compared to 0.4 for peptide **1**. This observation shows the importance of Phe-12 substitution for optimal *Candida* killing activity. The activity profile of the Phe-12-substituted peptide **3** well represents the divergent effects of Phe-12 on *Candida* and TNF-α**.** Relative to the nonsubstituted peptide, candidacidal activity of the sequence was greater, but potency in reducing TNF-α release was significantly less.

Influence of [D-Nal(2′)-7] α-MSH (6−13) on TNF-α Production in Vivo. As stated above, petide **2** [D-Nal-(2′)-7]-α-MSH (6−13), showed potent anti-TNF-α properties associated to substantial candidacidal effect. Therefore, this compound was tested for its capacity to inhibit TNF-α in plasma of endotoxin-treated rats. Data showed that the peptide was very efficient in reducing TNF-α in this in vivo model of septic shock (Figure 2). Indeed, the rise in circulating TNF-α in peptide **2**-treat-

ed animals was only 43% of the mean TNF-α concentration observed in blood of saline-treated controls ($p < 0.001$). Similar to in vitro experiments on human PBMC, the nonsubstituted fragment α-MSH (6−13) was significantly less effective than the DNal(2′)-7 substituted molecule ($p < 0.01$).

Truncation and Substitution Studies. To determine the relative importance of each amino acid residue in the [DNal(2′)-7, Phe-12]-α-MSH (6−13) sequence to kill *Candida* cells, systematic truncation studies at the N- and C-terminus and alanine substitions in the 6−9 "core" sequence were undertaken. Parallel experiments explored the anti-TNF-α activity of each peptide (Table 1).

His-6 deletion (peptide**4)** or its substitution with alanine (peptide **10)** reduced candidacidal activity to 51.4% and 62.8%, respectively. The anti-TNF-α activity of the peptides consensually dropped to <10% when histidine was deleted or substituted.

Further amino acid deletions resulted in peptides lacking both candidacidal and anti-TNF-α influences (peptides **5**−**7**). The Phe-12 substituted tripeptide Lys-Phe-Val (peptide **8**) had no candidacidal effect whereas the DNal(2′)-7 substituted "core" sequence His-DNal(2′)-Arg-Trp (peptide **9**) still maintained a small effect (43.0%).

Substitutions of DNal(2′)-7 and Trp-9 with alanine (peptide **11** and **13**) reduced substantially candidacidal and anti-TNF-α activity whereas substitution of Arg-8 (peptide **12**) only slightly altered the candidacidal effect of the peptide that remained remarkably high (95.8%).

Discussion

The data indicate that the α-MSH analogue [DNal-(2′)-7, Phe-12]-α-MSH (6−13) markedly inhibits *C. albicans* colony formation but its capacity to inhibit TNF-α release by endotoxin-stimulated human blood cells is not enhanced. This observation suggests that increase in antimicrobial properties does not always coexist with enhanced anti-cytokine activity. Indeed, DNal(2′)-7 and Phe-12 substitutions had distinct influences on α-MSH (6−13). Whereas replacement of Phe-7 with DNal(2′) increased both the candidacidal and the anti-TNF-α potential, substitution of Pro-12 with phenilalanine enhanced only the candidacidal effects.

Modifications of the Phe-7 residue were based on observations that this residue is critical for receptor binding and activation.[23,24,25] Indeed, substitution of Phe-7 with DPhe orginated the archetype α-MSH superpotent analogue [NLeu-4, DPhe-7]-α-MSH (1−13).[26] However, our previous research showed the DPhe-7 substitution does not result in peptides with very potent candidacidal influences.[18] On the contrary, certain DNal-(2′)-7 substituted peptides had enhanced activity against *Candida* cells.[18] The present research indicates that DNal(2′)-7 substitution markedly increases not only the candidacidal potential but also the anti-TNF-α properties of synthetic melanocortins. This observation is consistent with the agonistic activity of DNal-7-substituted peptides at the MC1R.[27] Indeed, the inhibitory activity exerted by α-MSH and synthetic melanocortins on TNF-α release by inflammatory cells is believed to occur through activation of the MC1R subtype.[28,29] Conversely, until the "melanocortin like" receptor in *Candida* is cloned and its structure elucidated, it is not possible to identify the precise type of interactions with melanocortin ligands.

The 6−9 His-Phe-Arg-Trp "core" sequence of melanocortins has long been considered essential for melanocortin receptor recognition and activation.[27] Alanine replacement studies in mouse B16 melanoma cells showed that when each amino acid in the 6−9 sequence was replaced by alanine there was a substantial decrease in receptor recognition and activation by α-MSH.[25] Results of the Ala-scan on peptide activity in *Candida* cells were very similar. Alanine substitutions, with the exception of Arg-8, reduced candidacidal influences, and even more clearly, diminished or abolished the anti-TNF-α properties. Similarly, truncation studies at bot the N- and the C-terminus indicated substantial or complete loss of candidacidal and anti-TNF-α activity.

A peculiar observation was that Arg-8 substitution with alanine (peptide **12**) only slightly altered the candidacidal effect of the peptide. Indeed, similar to Phe-7, Arg-8 replacement with alanine in melanocortin peptides causes marked decrease in ligand potency or even inability to stimulate the melanocortin receptors.[25,30,31] Several studies suggested that acidic residues present in TM2 and TM3 of the melanocortin receptors interact with the basic arginine residue of melanocortin ligands.[32,33] The observation that candidacidal influences remain virtually unaltered after Arg-8 replacement with alanine discloses a major difference between mammalian melanocortin receptors and the putative "melanocortin-like" receptor in *Candida*.

In conclusion, the data indicate that candidacidal and anti-cytokine influences of melanocortins can be separated through selective amino acid substitutions to produce peptides that are primarily candidacidal or anti-cytokine. More important for clinical practice, it is possible to obtain synthetic melanocortins that combine antimicrobial and antiinflammatory effects.

Experimental Section

Materials. N^{α}-Fmoc-protected amino acids, HBTU, HOBt, and Rink amide resin were purchased from Advanced ChemTech (Louisville, KY). For the N^{α}-Fmoc-protected amino acids, the following side chain protecting groups were used: Arg(N^{g}-Pbf), His(N^{im}-Trt), Trp(N^{in}-Boc), Lys(Boc), and Tyr(*t*Bu). Peptide synthesis solvents and reagents, as well as CH_3CN for HPLC, were reagent grade and were acquired from commercial sources and used without further purification unless otherwise noted. FAB-MS analyses were performed by MALDI. The purity of the finished peptides was checked by analytical RP-HPLC using a Shimadzu mod. CL-10AD VP system with a built-in diode array detector. In all cases, the purity of the finished peptides was greater than 95% as determined by these methods.

General Method for Peptide Synthesis and Purification. All peptides were synthesized by the solid-phase method of peptide synthesis and purified by RP-HPLC. The peptides were synthesized on 0.15 g each of Rink amide resin (substitution 0.7 mmol/g) by manual methods and by Advanced ChemTech automated peptide synthesizer 348 Ω using N^{α}-Fmoc chemistry and an orthogonal side chain protection strategy. The entire synthesis was performed under argon. The resin was first swollen in DCM/DMF (1:1) for 2 h and the following amino acids were then added to the growing peptide chain by stepwise addition of N^{α}-Fmoc-Val-OH, N^{α}-Fmoc-Pro-OH, N^{α}-Fmoc-Lys(Boc)-OH, N^{α}-Fmoc-Gly-OH, N^{α}-Fmoc-Trp-(Boc)-OH, N^{α}-Fmoc-Arg(N^{g}-Pbf)-OH, N^{α}-Fmoc-Phe-OH, and

N^{α}-Fmoc-His(N^{im}-Trt)-OH, using standard solid-phase methods. Each coupling reaction was achieved using a 3-fold excess of each of the amino acid, HBTU, and HOBt in the presence of a 6-fold excess of DIPEA for 1 h. Deprotection of the N^{α}-Fmoc group was carried out by treating the protected peptide resin with 25% piperidine solution in DMF (1 × 4 mL, 20 min). After each coupling and deprotection, the peptide resin was washed with DMF (3 × 4 mL), DCM (3 × 50 mL), and again with DMF. The peptide sequences were thus assembled by alternate cycles of coupling and deprotection. After coupling of the *N*-terminal amino acid, the *N*-terminal Fmoc group was deblocked as described above and the peptide-resin was thoroughly washed with DCM (4 × 25 mL) and dried under argon to yield dried peptide-resin.

The peptide-resin was then cleaved by treating with 4 mL of a solution of Et_3SiH (5%), water (5%), and *p*-thiocresol/*p*-cresol (0.1%, 1:1) in TFA with shaking at room temperature for 3 h. The resin was then removed from the solution by filtration, and the crude peptide was recovered by precipitation with cold anhydrous ethyl ether. Centrifugation at 1500*g* for 3 min followed by decantation of the supernatant ether and air-drying of the residue yielded the crude peptide as a white to pale beige colored amorphous solid.

Final peptide purification was achieved using a preparative RP-HPLC Vydac C18 (218TP1520, 15 μm). The peptide samples were injected onto the column at a concentration of 20−30 mg/mL in 20% aqueous CH_3CN and were eluted with a CH_3CN gradient (10 to 90%) over 40 min at a flow rate of 15.0 mL/min, with a constant concentration of TFA (0.1% v/v). The separations were monitored at 230 and 280 nm and integrated with a Shimadzu diode array detector mod. SPD-M10A VP dual wavelength absorbance detector model UV-D. Fractions corresponding to the major peak were collected, pooled, and lyophilized to yield the final peptides as pure (>95%) white solids. Amino acid analyses were carried out using a Pico-Tag Work Station. Lyophilized samples of peptides (50−1000 pmol) were hydrolyzed in heat-treated borosilicate tubes (4 × 50 mm) using the Pico-Tag Work Station (Waters-Millipore, Waltham, MA) for 1 h at 150 °C with 200 mL of 6 N HCl containing 1% phenol: a Pico-Tag column (3.9 × 15 mm) was employed to separate the amino acid derivatives. The analytical data and the amino acid analysis for each compound are presented in Supporting Information.

Organism and Culture Conditions. Five clinical isolates of *C. albicans* and the ATCC 24433 strain were obtained from the collection of the laboratory of Microbiology, Ospedale Maggiore di Milano. *Candida* cells were maintained on Sabouraud's agar slants and periodically transferred to Sabouraud's agar plates and incubated for 48 h at 28 °C. To prepare stationary growth phase yeast, a colony was taken from the agar plate and transferred into 5 mL of Sabouraud-dextrose broth and incubated for 48 h at 32 °C. Cells were centrifuged at 1000*g* for 10 min, and the pellet was washed twice with distilled water. Cells were counted and suspended in distilled water to obtain 10^7 yeast cells/mL. Viability, determined by the exclusion of 0.01% methylene blue, remained >98%.

Candidacidal Assay. The candidacidal influence of each peptide was determined by measuring colony forming units (CFU) in agar plates. This method was selected to ensure determination of even subtle changes in the number of organisms killed by each peptide. Tubes containing *C. albicans* 1×10^6 in 100 μL of distilled water were treated with 100 μL of each peptide (10^{-4} M final concentrations). Control tubes received 100 μL of distilled water. All the tests were run in triplicate on two independent experiments. After 2 h incubation at 37 °C, yeast suspensions from each vial were diluted with distilled water to obtain approximately 100 organisms/mL in control samples. A one milliliter aliquot from each tube was dispensed on blood agar plates and incubated for 48 h at 37 °C. The resultant CFU in test and control inocula were quantified by manual counting. The fungicidal activity of each peptide was calculated as follows:[100 − (CFU in test plates/CFU in control plates) × 100].

TNF-α Production by Endotoxin-Stimulated Human PBMC. Heparinized blood (20 mL) was obtained from five normal subjects. 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 (Gibco BRL, Paisley, UK) supplemented with 10 mM HEPES (Sigma), 2 mM L-glutamine (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 2.5×10^5 cells per well and incubated in 5% CO_2 atmosphere at 37 °C for 24 h in the presence of (a) medium alone; (b) medium plus the investigational peptide 10^{-4} M; (c) medium plus endotoxin from *Escherichia coli* 055:B5 (Sigma Chemical Co., St. Louis, MO) 1 ng/mL; (d) medium plus endotoxin 1 ng/mL plus the investigational peptide 10^{-4} M. After a 24 h-incubation, samples were centrifuged and supernatants separated and stored at −80 °C. TNF-α was measured using a specific immunoassay kit (Biosource International, Camarillo, CA). Viability in cell pellets was >95%, as measured by trypan blue exclusion.

Endotoxin-Induced Circulating TNF-α in Vivo. Male Wistar rats, 200−220 g, (Charles River, Calco, Italy) were maintained at the animal care facilities of Ospedale Maggiore di Milano, under standard temperature, humidity, and time-regulated light conditions. Water and food were provided ad libitum. Animals received care in compliance with the *Guide for the Care and Use of Laboratory Animals.*[34] Increase in circulating TNF-α was induced by iv injection of endotoxin 250 μg/kg (from *Escherichia coli* 055:B5, Sigma Chemical Co., St. Louis, MO) under light ether anesthesia. Endotoxin-treated rats ($n = 5$ per group) received iv injections of either (1) 1 mL of saline, or (2) [DNal(2′)-7, Pro-12]-α-MSH (6−13) 500 μg/kg dissolved in 1 mL of saline; or (3) the same amount of [Phe-7, Pro-12]-α-MSH (6−13). After 2 h, each rat was sacrificed under ether anesthesia. Blood was obtained from the inferior vena cava and immediately centrifuged; plasma was stored at −80 °C. TNF-α was measured using a specific immunoassay kit (Biosource International, Camarillo, CA).

Statistical Analysis. Data were analyzed using one-way analysis of variance 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 ± SD.

Appendix

Abbreviations used for amino acids and designation of peptides follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature in *J. Biol. Chem.* **1972**, *247*, 977−983. The following additional abbreviations are used: Boc, *tert*-butyloxycarbonyl; DCM, dichloromethane; DIPEA, *N,N*-diisopropylethylamine; DMF, *N,N*-dimethylformamide; Et_3SiH, triethylsilane; FAB-MS, fast-atom bombardment mass spectrometry; Fmoc, 9-fluorenylmethoxycarbonyl; HBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-(2-ethanesulfonic acid); HOBt, *N*-hydroxybenzotriazole; LPS, lipolysaccharide; NDP-α-MSH, (Nle-4,DPhe-7)-α-MSH; PBMC, peripheral blood mononuclear cells; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; POMC, proopiomelanocortin; RP-HPLC, reversed-phase high performance liquid chromatography; *t*Bu, *tert*-butyl; TFA, trifluoroacetic acid; TNF-α, tumor necrosis factor-α; Trt, triphenylmethyl (trityl). Amino acid symbols denote the *L*-configuration unless indicated otherwise.

Supporting Information Available: Physicochemical properties and amino acid analysis of the peptides. This

material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Eberle, A. *The melanotropins*; Karger: Basel, 1988.
(2) Lipton, J. M.; Catania, A. Antiinflammatory actions of the neuroimmunomodulator α-MSH. *Immunol. Today* **1997**, *18*, 140−145.
(3) Catania, A.; Gatti, S.; Colombo, G.; Lipton, J. M. Targeting melanocortin receptors as a novel strategy to control inflammation. *Pharmacol. Rev.* **2004**, *56*, 1−29.
(4) Luger, T. A.; Brzoska, T.; Scholzen, T. E.; Kalden, D. H.; Sunderkotter, C. et al. The role of α-MSH as a modulator of cutaneous inflammation. *Ann. N. Y. Acad. Sci.* **2000**, *917*, 232−238.
(5) Ellacott, K. L.; Cone, R. D. The central melanocortin system and the integration of short- and long-term regulators of energy homeostasis. *Recent Prog. Horm. Res.* **2004**, *59*, 395−408.
(6) Heisler, L. K.; Cowley, M. A.; Tecott, L. H.; Fan, W.; Low, M. J. et al. Activation of central melanocortin pathways by fenfluramine. *Science* **2002**, *297*, 609−611.
(7) Li, S. J.; Varga, K.; Archer, P.; Hruby, V. J.; Sharma, S. D. et al. Melanocortin antagonists define two distinct pathways of cardiovascular control by α- and γ-melanocyte-stimulating hormones. *J. Neurosci.* **1996**, *16*, 5182−5188.
(8) Dunbar, J. C.; Lu, H. Proopiomelanocortin (POMC) products in the central regulation of sympathetic and cardiovascular dynamics: studies on melanocortin and opioid interactions. *Peptides* **2000**, *21*, 211−217.
(9) Versteeg, D. H.; Van Bergen, P.; Adan, R. A.; De Wildt, D. J. Melanocortins and cardiovascular regulation. *Eur. J. Pharmacol.* **1998**, *360*, 1−14.
(10) Abdel-Malek, Z.; Scott, M. C.; Suzuki, I.; Tada, A.; Im, S. et al. The melanocortin-1 receptor is a key regulator of human cutaneous pigmentation. *Pigment Cell Res.* **2000**, *13 Suppl. 8*, 156−162.
(11) Hadley, M. E.; Hruby, V. J.; Blanchard, J.; Dorr, R. T.; Levine, N. et al. Discovery and development of novel melanogenic drugs. Melanotan-I and -II. *Pharm. Biotechnol.* **1998**, *11*, 575−595.
(12) Slominski, A.; Wortsman, J.; Luger, T.; Paus, R.; Solomon, S. Corticotropin releasing hormone and proopiomelanocortin involvement in the cutaneous response to stress. *Physiol. Rev.* **2000**, *80*, 979−1020.
(13) Cutuli, M.; Cristiani, S.; Lipton, J. M.; Catania, A. Antimicrobial effects of α-MSH peptides. *J. Leukocyte Biol.* **2000**, *67*, 233−239.
(14) Catania, A.; Cutuli, M.; Garofalo, L.; Carlin, A.; Airaghi, L. et al. The neuropeptide α-MSH in host defense. *Ann. N. Y. Acad. Sci.* **2000**, *917*, 227−231.
(15) Wikberg, J. E.; Muceniece, R.; Mandrika, I.; Prusis, P.; Lindblom, J. et al. New aspects on the melanocortins and their receptors. *Pharmacol. Res.* **2000**, *42*, 393−420.
(16) Haskell-Luevano, C.; Miwa, H.; Dickinson, C.; Hruby, V. J.; Yamada, T. et al. Binding and cAMP studies of melanotropin peptides with the cloned human peripheral melanocortin receptor, hMC1R. *Biochem. Biophys. Res. Commun.* **1994**, *204*, 1137−1142.
(17) Mountjoy, K. G.; Robbins, L. S.; Mortrud, M. T.; Cone, R. D. The cloning of a family of genes that encode the melanocortin receptors. *Science* **1992**, *257*, 1248−1251.
(18) Grieco, P.; Rossi, C.; Colombo, G.; Gatti, S.; Novellino, E. et al. Novel α-melanocyte stimulating hormone peptide analogues with high candidacidal activity. *J. Med. Chem.* **2003**, *46*, 850−855.
(19) Skottner, A.; Post, C.; Ocklind, A.; Seifert, E.; Liutkevicius, E. et al. Antiinflammatory potential of melanocortin receptor-directed drugs. *Ann. N. Y. Acad. Sci.* **2003**, *994*, 84−89.
(20) Getting, S. J. Melanocortin peptides and their receptors: new targets for antiinflammatory therapy. *Trends Pharmacol. Sci.* **2002**, *23*, 447−449.
(21) Szardenings, M.; Muceniece, R.; Mutule, I.; Mutulis, F.; Wikberg, J. E. New highly specific agonistic peptides for human melanocortin MC(1) receptor. *Peptides* **2000**, *21*, 239−243.
(22) Muceniece, R.; Krigere, L.; Suli-Vargha, H.; Wikberg, J. E. Effects of α-melanotropin C-terminal tripeptide analogues on macrophage NO production. *Peptides* **2003**, *24*, 701−707.
(23) Hruby, V. J.; Wilkes, B. C.; Hadley, M. E.; Al-Obeidi, F.; Sawyer, T. K. et al. α-Melanotropin: the minimal active sequence in the frog skin bioassay. *J. Med. Chem.* **1987**, *30*, 2126−2130.
(24) Castrucci, A. M.; Hadley, M. E.; Sawyer, T. K.; Wilkes, B. C.; al-Obeidi, F. et al. α-Melanotropin: the minimal active sequence in the lizard skin bioassay. *Gen. Comp. Endocrinol.* **1989**, *73*, 157−163.
(25) Sahm, U. G.; Olivier, G. W.; Branch, S. K.; Moss, S. H.; Pouton, C. W. Synthesis and biological evaluation of α-MSH analogues substituted with alanine. *Peptides* **1994**, *15*, 1297−1302.
(26) Sawyer, T. K.; Sanfilippo, P. J.; Hruby, V. J.; Engel, M. H.; Heward, C. B. et al. 4-Norleucine, 7-D-phenylalanine-α-melanocyte-stimulating hormone: a highly potent α-melanotropin with ultralong biological activity. *Proc. Natl. Acad. Sci. U. S.A.* **1980**, *77*, 5754−5758.
(27) Holder, J. R.; Haskell-Luevano, C. Melanocortin ligands: 30 years of structure−activity relationship (SAR) studies. *Med. Res. Rev.* **2004**, *24*, 325−356.
(28) Ignar, D. M.; Andrews, J. L.; Jansen, M.; Eilert, M. M.; Pink, H. M. et al. Regulation of TNF-α secretion by a specific melanocortin-1 receptor peptide agonist. *Peptides* **2003**, *24*, 709−716.
(29) Taherzadeh, S.; Sharma, S.; Chhajlani, V.; Gantz, I.; Rajora, N. et al. α-MSH and its receptors in regulation of tumor necrosis factor-α production by human monocyte/macrophages. *Am. J. Physiol.* **1999**, *276*, R1289−1294.
(30) Grieco, P.; Balse-Srinivasan, P.; Han, G.; Weinberg, D.; MacNeil, T. et al. Synthesis and biological evaluation on hMC3, hMC4 and hMC5 receptors of γ-MSH analogs substituted with L-alanine. *J. Pept. Res.* **2002**, *59*, 203-210.
(31) Holder, J. R.; Xiang, Z.; Bauzo, R. M.; Haskell-Luevano, C. Structure−activity relationships of the melanocortin tetrapeptide Ac−His-DPhe-Arg-Trp-NH2 at the mouse melanocortin receptors. Part 3: modifications at the Arg position. *Peptides* **2003**, *24*, 73−82.
(32) Yang, Y. K.; Fong, T. M.; Dickinson, C. J.; Mao, C.; Li, J. Y. et al. Molecular determinants of ligand binding to the human melanocortin-4 receptor. *Biochemistry* **2000**, *39*, 14900−14911.
(33) Yang, Y.; Dickinson, C.; Haskell-Luevano, C.; Gantz, I. Molecular basis for the interaction of [Nle4,D-Phe7]melanocyte stimulating hormone with the human melanocortin-1 receptor. *J. Biol. Chem.* **1997**, *272*, 23000−23010.
(34) Commission on Life Sciences, N. R. C. *Guide for the Care and Use of Laboratory Animals*; The National Academy Press: Washington, DC, 1996.

JM040890J