Sebocytes

Expression and Signaling in Human

Mediated Cytokine

− β

Suppresses IL-1

−

- Melanocyte

α

KdPT, a Tripeptide Derivative of α-

-Melanocyte-Stimulating Hormone,

Suppresses IL-1 β-Mediated Cytokine

Expression and Signaling in Human

Sebocytes

Arianna Mastrofrancesco, Agatha Kokot, Alex Eberle, Nicholas C. J. Gibbons, Karin U. Schallreuter, Elwira Strozyk, Mauro Picardo, Christos C. Zouboulis, Thomas A. Luger and Markus Böhm

J Immunol 2010; 185:1903-1911; Prepublished online 7 July 2010;

doi: 10.4049/jimmunol.0902298

http://www.jimmunol.org/content/185/3/1903

Supplementary Material

http://www.jimmunol.org/content/suppl/2010/07/06/jimmunol.0902298.DC1

References

This article cites 44 articles, 10 of which you can access for free at:

http://www.jimmunol.org/content/185/3/1903.full#ref-list-1

Why The JI? Submit online.

• Rapid Reviews! 30 days* from submission to initial decision

• No Triage! Every submission reviewed by practicing scientists

• Fast Publication! 4 weeks from acceptance to publication

*average

Subscription

Information about subscribing to The Journal of Immunology is online at:

http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
KdPT, a Tripeptide Derivative of α-Melanocyte–Stimulating Hormone, Suppresses IL-1β–Mediated Cytokine Expression and Signaling in Human Sebocytes

Arianna Mastrofrancesco,*1 Agatha Kokot,* Alex Eberle,7,8 Nicholas C. J. Gibbons,8 Karin U. Schallreuter,5 Elwira Strozyk,*6 Mauro Picardo,‖ Christos C. Zouboulis,# Thomas A. Luger,* and Markus Böhm*,1

Acne is the most common inflammatory skin disease in which IL-1 plays a central role. Although α-melanocyte–stimulating hormone has immunomodulatory effects, its usefulness as an anti-inflammatory agent in acne is hampered owing to its lipid- and pigment-inducing effects via activation of melanocortin receptors (MC-Rs). We used the immortalized human sebocyte line SZ95 as an in vitro model to investigate the anti-inflammatory potential of KdPT, a tripeptide derivative of the C-terminal end of α-melanocyte–stimulating hormone. KdPT potently suppressed IL-1β–induced IL-6 and IL-8 expression. Mechanistically, KdPT decreased IL-1β–mediated ΩBox degradation, reduced nuclear accumulation of p65, and attenuated DNA binding of NF-κB. Moreover, KdPT reduced IL-1β–mediated generation of intracellular reactive oxygen species, which contributed to IL-1β–mediated cytokine induction. KdPT also reduced cell surface binding of fluorochrome-labeled IL-1β in SZ95 sebocytes. Analysis of the crystal structure of the complex between IL-1β/IL-1R type 1 (IL-1RI), followed by computer modeling of KdPT and subsequent modeling of the peptide receptor complex with the crystal structure of IL-1RI via manual docking, further predicted that the tripeptide, through several H-bonds and one hydrophobic bond, interacts with the IL-1RI. Importantly, KdPT did not bind to MC-1Rs, as demonstrated by blocking experiments with a peptide analog of Agouti signaling protein and by binding assays using MC-1R–expressing B16 melanoma cells. Accordingly, KdPT failed to induce melanogenesis. Our data demonstrate a promising anti-inflammatory potential of KdPT and point toward novel future directions in the treatment of acne—as well as of various other IL-1–mediated inflammatory diseases—with this small molecule. The Journal of Immunology, 2010, 185: 1903–1911.
therapeutic exploitation of α-MSH as an anti-inflammatory agent in patients with acne is problematic for several reasons because natural and synthetic melanocortin peptides such as [Nle^4, D-Phe^7] α-MSH (NDP-α-MSH) or adrenocorticotropic hormone directly increase lipogenesis in primary human sebocytes (20). Moreover, the melanotropic (pigment-inducing) activity of α-MSH analogs containing the central pharmacophor may be a limiting factor for their use, especially in Caucasian white-skinned patients.

KOPT (Lys^5-Pro-Thr) is a derivative of the C-terminal sequence of α-MSH in which the second amino acid, Pro, is replaced by its D-enantiomer and the third amino acid, Val, is exchanged for Thr. It is of interest that KPT, the L-enantiomer, is colinear with human IL-1β193–195. Originally, KOPT was reported to attenuate the IL-1β-mediated hyperalgesic response in a rat-paw pressure test (21, 22). KOPT, however, does not appear to act as a simple or uniform IL-1β antagonist because the peptide did not have antiproteic activity against IL-1β in the rabbit pyrogen test (21). It was also inactive in the IL-1β-evoked relaxation of rabbit isolated mesenteric artery (23). So far, little research on KOPT has been conducted; consequently, the anti-inflammatory potential of this substance remains largely elusive.

In this study, we investigated the anti-inflammatory potential of KOPT on SZ95 sebocytes, which show the morphologic, phenotypic, and functional characteristics of primary human sebocytes (24). Because acne does not exist outside the human system and no animal models for this disease are available, this immortalized human sebocyte cell line has evolved as an important research tool in acne research to study the influence of various immune mediators and neuropeptides (25).

Our data provide compelling evidence for potent anti-inflammatory effects of KOPT on IL-1β–mediated IL-6 and IL-8 expression. These effects of KOPT were not mediated by binding to the MC-1R. We demonstrate that KOPT suppresses IL-1β–mediated NF-κB activation, possibly via interaction with the third Ig-like C2 domain of IL-1R type I (IL-1R1) and via reduced formation of oxidative stress. Because these anti-inflammatory effects of KOPT are not based on MC-1R binding, they cannot enhance melanogenesis. Hence, it is tempting to propose major potential for the tripeptide in the treatment of inflammatory diseases, including acne.

Materials and Methods

Cell culture

SZ95 sebocytes (25) were maintained in Sebomed basal medium supplemented with 5 ng/ml human epidermal growth factor, 10% FCS (both from Biochrom, Berlin, Germany), 1% l-glutamine, 1% penicillin/streptomycin, and 1 mM CaCl2 in a humidified atmosphere containing 5% CO2 at 37˚C. Human melanocytes obtained from Tepu-bio (Portland, OR) were routinely grown in MGM-M2 plus all supplements (Cascade Biologics, Portland, OR). Mouse B16-F1 melanoma cells (26) were cultured in MEM supplemented with 10% heat-inactivated FCS, 2 mM l-glutamine, 1% nonessential amino acids, 1% vitamin solution, 50 IU/ml penicillin, and 50 μg/ml streptomycin (conditions as described for sebocytes).

Conventional and real-time RT-PCR

SZ95 sebocytes were seeded into 6-cm or 9-cm tissue culture dishes at a density of 2 × 10^5 cells per dish. After serum deprivation as outlined above, cells were washed three times with PBS, followed by simultaneous treatment with 5 μg/ml 5-(and 6-)chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (CM-H2DCFDA) (Molecular Probes, Karlsruhe, Germany), IL-1β (1 ng/ml), or IL-1β plus KOPT (10−6 M or 10−8 M) for 20 min in PBS with glucose (5 mM) at 37˚C in the dark. After treatment, cells were trypsinized, centrifuged, and resuspended in PBS with glucose (5 mM). Oxidative conversion of CM-H2DCFDA to the fluorescent product in living cells was assessed by flow cytometry using a FACSCalibur (BD Biosciences, San Jose, CA), equipped with a 488-nm laser. A total of 1 × 10^4 cells from each sample was acquired. CellQuest Pro software (BD Biosciences) was used for analyzing the data. The median of FL-1 channel of fluorescence was used as the parameter to evaluate the intracellular oxidative stress because it matches the maximal number of cells with the highest fluorescence.

Western immunoblotting

Serum-deprived SZ95 sebocytes were stimulated with IL-1β (1 ng/ml) or IL-1β plus KOPT (10−6 M) for 15 min. Cells were then trypsinized and washed with ice-cold PBS. The resulting pellets were resuspended into ice-cold lysis buffer (10 mM HEPES, pH 7.3, 0.1 mM EDTA; 10 mM NaCl; 1 mM DTT; 5% glycerol; 50 mM NaF; 10 mM sodium molybdate) containing the Complete Protease Inhibitor Set (Roche, Mannheim, Germany), followed by spectrophotometric protein measurement. Identical amounts of protein (50 μg/fane) were resolved on 10% SDS-PAGE. The proteins were electrophoretically transferred to nitrocellulose membranes. After blocking with 2% Blocking Reagent (Roche), membranes were incubated with a mAb against ioeBα (1:500; IMGENEX, San Diego, CA) in 1% Blocking Reagent overnight at 4˚C. After washing, membranes were incubated with an anti-mouse HRP-conjugated Ab (1:5000 dilution) (Amersham Pharmacia, Piscataway, NJ) in 1% Blocking Reagent for 1 h at room temperature. Bound Abs were detected using the ECL Plus chemiluminescent reagent (Amersham-Pharmacia). Identical protein loading was confirmed by stripping the blot and reprobing it with an anti–α-tubulin Ab (Oncogene, San Diego, CA).

Immunofluorescence analysis

SZ95 sebocytes were seeded into eight-well chamber slides and were fixed with methanol for 30 min at −20˚C. Nonspecific binding was blocked with 5% donkey serum for 1 h at room temperature. Cells were then incubated for 1 h with the rabbit polyclonal Ab against α–NF-κB p65 (Santa Cruz Biotechnology, San Diego, CA) (1 μg/ml). Bound Abs were visualized with a donkey anti-rabbit (1:1500) Ab coupled to Texas Red (Dianova, Hamburg, Germany). For nuclear staining, 4′,6-diamidino-2-phenylinde
(DAPI) (Fluka, Taufkirchen, Germany) was used. Cells were imaged and examined with a fluorescence microscope (Carl Zeiss, Oberkochen, Germany). Red fluorescence was excited using the TX3 filter (530–585 nm); for DAPI staining an excitation wavelength of 365 nm was used and emission was measured at 615 nm.

EMSA

SZ95 sebocytes were seeded into 10-cm Ø tissue culture dishes at a density of 5 \times 10^6 cells. Following deprivation, cells were stimulated for 15, 30, or 60 min with IL-1β (1 ng/ml), KDPT (10^{-6} M), or both agents. Cells were then washed with ice-cold PBS, scraped in low-salt buffer A consisting of 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSE, and the Complete Protease Inhibitor Set, and then incubated on ice for 20 min. The lysates were subsequently passed 10 times through a 26G1/2-gauge needle. After centrifugation, nuclear pellets were resuspended in high-salt buffer C (20 mM HEPES, pH 7.9; 400 mM NaCl; 1 mM EDTA; 1 mM EGTA; 1mM DTT; and protease inhibitors, as mentioned above), followed by intense shaking at 4˚C for 30 min. Nuclear lysates were cleared by centrifugation and the protein content determined by the DC Protein Assay Kit (Bio-Rad, Hercules, CA). The NF-κB consensus oligonucleotide (Santa Cruz Biotechnology) was end labeled using [γ^{32}P]ATP and T4 polynucleotide kinase (Fermentas), followed by column purification (QIAquick Nucleotide Removal Kit, Qiagen). Binding reactions were carried out in a 20-μl volume of 4 mM HEPES (pH 7.5), 10 mM KCl, 0.5 mM MgCl2, 4% (w/v) Ficoll, 0.2 mM DTT containing 14 μg protein extract, 2 μg poly [dI-dC], 2 μg BSA, and 60,000 cpm of 32P-labeled NF-κB consensus oligonucleotide. Reaction samples were incubated for 20 min at room temperature. For supershift assays, EMSA samples were incubated with 2 μg of the respective supershift Ab recognizing p65 or p50 (Santa Cruz Biotechnology) for 20 min at room temperature prior to addition of 32 P-labeled NF-κB oligonucleotide. Reaction samples were separated on a 4.5% native PAGE at 150 V for 2.5 h and detected by autoradiography. α-MSH binding assays

Competition binding experiments were performed in 96-well U-bottom microplates (BD Biosciences, Heidelberg, Germany), each well containing 100 μl murine B16-F1 or human SZ95 sebocyte cell suspensions adjusted to 4 \times 10^6 cells/ml. The binding medium consisted of MEM with Earle’s salts, 0.2% BSA, and 0.3 mM 1,10-phenanthroline. Triplicates of competitor peptide solution (50 μl), yielding a final concentration ranging from 1 \times 10^{-4} to 1 \times 10^{-12} M, were added, followed by the addition of 50,000 cpm [125I]NDP-α-MSH in 50 μl to each well (29). The incubation conditions were 15˚C for 3 h for B16-F1 cells and 15˚C for 3 h or 37˚C for 2 h for SZ95 cells. The reaction was stopped by placing the plates on ice for 10 min. The cell-bound radioactivity was collected on filters (Unifilter-96 GF/B; Packard, Meriden, CT) by use of a cell harvester, and the radioactivity was counted on a TopCount scintillation counter (Packard). The IC50 values were calculated with Prism software (GraphPad Software, San Diego). Determination of melanin

The potential melanotropic in vitro effect of KDPT at concentrations ranging from 10^{-6} to 10^{-10} M versus NDP-α-MSH (10^{-8} M) as a positive control was assayed on B16-F1 melanoma cells seeded at a density of 2500 cells per well in 96-well tissue culture plates. The melanin content in the supernatants of the cells after 72 h was determined photometrically at 405 nm, as described previously (30).

FIGURE 1. Effects of IL-1β and KDPT on IL-6 and IL-8 expression in SZ95 sebocytes. A and B, Cells were stimulated with different doses of IL-1β, as indicated, for 8 h. Relative mRNA levels of IL-6 and IL-8 were determined by real-time RT-PCR. n = 3; *p < 0.05; **p < 0.01 versus nontreated cells. C and D, KDPT at doses indicated was coincubated with IL-1β (1 ng/ml) for 8 h followed by real-time RT-PCR analysis of IL-6 and IL-8. n = 3; ***p < 0.001 versus IL-1β-treated cells. E and F, SZ95 sebocytes were stimulated with IL-1β (1 ng/ml) in the presence or absence of KDPT for 12 h. IL-6 and IL-8 amounts in cell culture supernatants were determined by ELISA. n = 3; ***p < 0.001 versus IL-1β-treated cells.
IL-1β–fluorochrome cell surface binding studies

Human rIL-1β (eBioscience, Frankfurt, Germany), 25 μg, was labeled with Alexa Fluor 488, using a commercially available protein labeling kit (Invitrogen, Darmstadt, Germany). SZ95 sebocytes were then seeded into 3.5-cm Ø tissue culture dishes at a density of $2 \times 10^5$ cells per dish. After serum deprivation, as described above, cells were washed with PBS, trypsinized, centrifuged, and transferred to FACS tubes. For the labeling reaction, cells were incubated on ice for 15 min with Alexa Fluor 488-labeled IL-1β. KDPT (10^{-7} M) or unlabeled IL-1β (1 ng/ml) was preincubated for 15 min. Cells were finally washed twice with ice-cold PBS to remove unbound molecules. Cell-bound fluorochrome-labeled IL-1β was visualized by flow cytometry, using the FACSCalibur, as outlined above.

Computer modeling

The crystal structures of the complex between human IL-1β and IL-1RI were obtained from the Protein Data Bank (accession code 11TB) (31). The resulting structures were analyzed by Deep View (Swiss Institute of Bioinformatics, Lausanne, Switzerland). Modeling and docking of the tripeptide KDPT to the receptor was followed by analysis of the resulting complex using HyperChem (Hypercube, Gainesville, FL). Figures are presented using both HyperChem and DeepView.

Data analysis

Real-time RT-PCR analysis, ELISAs, and melanin assays were performed in at least three independent experiments. Data were then calculated as means

Table I. KDPT-mediated inhibition (%) of IL-1β–induced IL-6 and IL-8 mRNA expression

<table>
<thead>
<tr>
<th>Condition</th>
<th>IL-6</th>
<th>IL-10</th>
<th>IL-1β, 0.1 ng/ml</th>
<th>+ KDPT 10^{-8} M</th>
<th>IL-6</th>
<th>IL-10</th>
<th>IL-1β, 1 ng/ml</th>
<th>+ KDPT 10^{-6} M</th>
<th>IL-6</th>
<th>IL-10</th>
<th>IL-1β, 10 ng/ml</th>
<th>+ KDPT 10^{-8} M</th>
</tr>
</thead>
</table>
| IL-1β, 0.1 ng/ml   | -41.06 ± 4.39 | -57.48 ± 0.87 | -53.37 ± 0.94 | -61 ± 9.77
| IL-1β, 1 ng/ml    | -90.85 ± 2.46 | -90.52 ± 2.21 | -91.71 ± 0.84 | -92.54 ± 0.97
| IL-1β, 10 ng/ml   | -65.58 ± 1.8 | -70.1 ± 4.91 | -69.6 ± 0.59 | -41.7 ± 1.71
| IL-1β, 10 ng/ml   | -93.97 ± 0.37 | -95.36 ± 0.65 | -94.06 ± 0.1 | -64.6 ± 0.98
| IL-1β, 10 ng/ml   | -13.02 ± 3.16 | -19.53 ± 8.65 | -15.59 ± 9.77 | -2.33 ± 9.06
| IL-1β, 10 ng/ml   | -38.22 ± 1.33 | -0.95 ± 3.25 | -6.03 ± 7.53 | -0.97 ±

*Non-significant compared with IL-1β.

FIGURE 2. KDPT suppresses cytokine expression/signaling in sebocytes

A. SZ95 sebocytes were stimulated with 1 ng/ml IL-1β, 10^{-6} M KDPT, both agents, or were left untreated. Nuclear extracts were generated after different time points of stimulation, as indicated, followed by EMSA. The NF-κB subunit composition of the detected DNA–protein complexes was identified by incubating nuclear extracts from IL-1β–stimulated cells with an anti-p65 or anti-p50 NF-κB Ab. Depicted is a representative set of three independently performed experiments with similar results. Lanes 1, 5, and 9, nontreated; lanes 2, 6, and 10, KDPT; lanes 3, 7, and 11, IL-1β; lanes 4, 8, and 12, KDPT + IL-1β. B. KDPT reduces IL-1β–mediated nuclear accumulation of p65. SZ95 sebocytes were treated with IL-1β (1 ng/ml), KDPT (10^{-6} M) plus IL-1β, or were left untreated for 30 min. p65 was detected by immunofluorescence analysis with an Ab against p65 (red signal) in combination with nuclear staining using DAPI (blue). Depicted is a representative set of n = 3 independent experiments with similar results. Original magnification ×400. C. KDPT attenuates IL-1β–induced IkBα degradation. SZ95 sebocytes were treated with IL-1β (1 ng/ml), KDPT (10^{-8} M), both substances, or were left untreated for 15 min. Cytoplasmic extracts were generated and subjected to Western immunoblotting using an anti-IκBα Ab. Equal protein loading was confirmed by stripping and reprobing the blot with an anti-tubulin Ab.
Results

**KoPT suppresses IL-1β–induced IL-6 and IL-8 expression in SZ95 sebocytes**

We first investigated the effect of KoPT on IL-1β–induced IL-6 and IL-8 mRNA expression by real-time RT-PCR. IL-1 via activating receptor subtype I (IL-1–RI) is a well-known inducer of both cytokines (10, 11). However, the impact of IL-1β on IL-6 and IL-8 expression in SZ95 sebocytes has not been examined in detail. IL-1β treatment for 8 h led to a dose-dependent increase in the mRNA amounts of both cytokines (Fig. 1A, B). For all subsequent functional studies with KoPT, a dose of IL-1β at 1 ng/ml was used.

Incubation of SZ95 sebocytes with various doses of KoPT (10−6 M–10−10 M) per se did not lead to any detectable changes in the relative mRNA amounts of both proinflammatory cytokines (Supplemental Fig. 1). In contrast, KoPT dose-dependently and significantly reduced IL-6 mRNA and IL-8 mRNA expression when coincubated with IL-1β (Fig. 1C, D). In fact, the tripeptide at 10−4, 10−6, and 10−8 M suppressed IL-1β–induced IL-6 and IL-8 mRNA expression almost to basal levels (calculated IC50 for IL-6 mRNA suppression: ∼10−6 M; for IL-8 mRNA suppression: <10−10 M). Dose-kinetic studies employing different IL-1β doses further revealed that the attenuating effect of KoPT depends on the concentration of coincubated IL-1β. Although, at IL-1β doses of 0.1 and 1 ng/ml KoPT at all tested concentrations significantly suppressed IL-6 and IL-8 mRNA expression, a suppressive effect in the presence of 10 ng/ml IL-1β could be achieved only at 10−6 or 10−8 M of the tripeptide (Table I).

The suppressive effect of KoPT on IL-6 and IL-8 mRNA expression in SZ95 sebocytes was subsequently confirmed at the protein level, using ELISA. As expected, stimulation with IL-1β resulted in a marked increase in the secreted amounts of IL-6 and IL-8. In accordance with the mRNA expression data, KoPT significantly and dose-dependently attenuated the inductive effect of IL-1β on the protein secretion of both cytokines (Fig. 1E, F).

KoPT attenuates IL-1β–mediated NF-κB signaling in SZ95 sebocytes

To determine the molecular mechanism of the suppressive effect of KoPT on IL-1β–mediated cytokine expression in SZ95 sebocytes, we next analyzed the NF-κB pathway, which is well established to be activated by IL-1β.

First, SZ95 sebocytes were treated with IL-1β alone or in the presence of KoPT at different time points. Nuclear extracts were isolated, and NF-κB–DNA binding activity was assessed by EMSA. In control cells, low NF-κB binding activity was detected, which was strongly enhanced after treatment with IL-1β (1 ng/ml) for 15, 30, and 60 min (Fig. 2A). Supershift experiments employing anti-p65 and anti-p50 Abs disclosed that the IL-1β–induced NF-κB–DNA binding complexes consist of p65/p65 homodimers and p65/p50 heterodimers. In accordance with the lack of any effect on IL-6 and IL-8 expression, KoPT (10−6 M) alone did not elicit any detectable effect on NF-κB DNA binding. Cotreatment of cells with IL-1β plus KoPT, however, attenuated DNA binding of NF-κB, presumably owing to reduced generation of p65/p50 homodimers (Fig. 2A).

The KoPT-mediated attenuation of the NF-κB pathway induced by IL-1β was confirmed by immunofluorescence analysis of p65 in SZ95 sebocytes. In nontreated cells, p65 immunostaining excluded the nuclei in virtually all cells. Instead, a diffuse cytoplasmic and perinuclear staining was detectable (Fig. 2B, left panel). Stimulation with IL-1β (1 ng/ml) for 30 min resulted in a clear-cut change of p65 immunostaining from the cytoplasm to the nucleus in the majority of cells, as shown by double staining with an anti-p65 Ab and the nuclear tracker DAPI (Fig. 2B, middle panel). Costimulation with IL-1β plus KoPT (10−6 M), in contrast, reduced the number of p65-expressing nuclei (Fig. 2B, right panel).

**FIGURE 3.** KoPT reduces intracellular ROS accumulation, which is essential for IL-1β–mediated IL-6 and IL-8 induction in SZ95 sebocytes. SZ95 sebocytes were stimulated with IL-1β (1 ng/ml) alone or in combination with KoPT (10−6 M). After 20 min, ROS production was determined by cytofluorimetric analysis using CM-H2DCFDA (5 μM) as a fluorescent probe. A, The diagram depicts a representative experiment of three independently performed experiments with identical results. B and C, SZ95 sebocytes were pretreated with MnCpx3 (10 μM). IL-6 and IL-8 mRNA levels were determined by real-time RT-PCR. n = 3; **p < 0.01 versus IL-1β-treated cells.
sebocytes with nuclear p65 staining, compared with cells exposed to IL-1β alone (Fig. 2B, right panel).

To further elucidate the molecular mechanism of KoPT-mediated attenuation of NF-κB activation by IL-1β, we finally performed Western immunoblotting of IκBα in cytoplasmic extracts of SZ95 sebocytes. NF-κB activation is triggered by phosphorylation of its inhibitor protein IκBα on Ser-32 and Ser-36 (32). As a result, IκBα dissociates from NF-κB in the cytoplasm and is degraded by the proteasome. As illustrated in Fig. 2C, treatment of SZ95 with IL-1β (1 ng/ml) for 15 min resulted in reduced protein amounts of IκBα, compared with unstimulated cells, indicating enhanced protein degradation. Although KoPT alone did not have any effect, cotreatment with IL-1β plus KoPT partially abrogated the cytokine-induced effect on IκBα degradation (Fig. 2C).

**KoPT reduces intracellular oxidative stress in IL-1β–treated SZ95 sebocytes**

Because intracellular generation of reactive oxygen species (ROS) plays an important role in IL-1β–induced NF-κB activation (33), we hypothesized that KoPT may suppress IL-1β–mediated signaling by suppressing intracellular ROS generation. SZ95 sebocytes were thus loaded with the ROS-detecting fluoroprobe CM-H2DCFDA and stimulated with IL-1β (1 ng/ml) alone or together with KoPT (10⁻⁶ M). As shown by FACS analysis, IL-1β stimulation led to a rightward shift in the mean fluorescence intensity, indicating increased amounts of intracellular ROS, compared with amounts in nontreated cells (Fig. 3A). KoPT alone did not have any effects (data not shown). In contrast, KoPT reduced IL-1β–induced ROS production, as demonstrated by a marked leftward shift in the mean fluorescence intensity, compared with IL-1β alone (Fig. 3A). In a series of three independent experiments, this KoPT-mediated decrease in IL-1β–induced intracellular ROS production was −30 ± 2.7% (p < 0.001).

To confirm the role of oxidative stress as an intracellular signaling event during IL-1β–mediated proinflammatory cytokine expression in SZ95 sebocytes, we pretreated the cells with the Mn complex of 7-hydroxy-flavone (MnCpx3). MnCpx3 was previously shown to exhibit potent superoxide dismutase activity and protection against lipid peroxidation in vitro (34). MnCpx3 significantly suppressed IL-1β–mediated mRNA expression of both IL-6 and IL-8 in SZ95 sebocytes (Fig. 3B, 3C).

**KoPT does not bind to MC-1R and has no melanotrophic activity**

In the next set of experiments, we determined whether KoPT mediates its biological effects via binding to MC-1Rs expressed by SZ95 sebocytes. In accordance with previous data (19), MC-1R mRNA was detectable by RT-PCR in these cells (data not shown). However, blocking experiments with a peptide analog of Agouti signaling protein (ASIP) acting as an MC1R/MC-4R antagonist (35, 36) did not result in abrogation of the suppressive effect of KoPT on IL-1β–induced IL-6 or IL-8 mRNA expression (Fig. 4A, 4B). To clearly exclude the involvement of an MC-1R–mediated action of KoPT in SZ95 sebocytes, radioligand binding assays with radio-labeled ¹²⁵I-NDP–α-MSH were then performed. SZ95 sebocytes failed to show any specific NDP-α–MSH radioligand binding, whereas B16-F1 melanoma cells used as positive control readily revealed specific binding of the tracer (Fig. 4C). These data indicate that in SZ95 sebocytes, binding sites for α-MSH are below the detection limit of classical radioligand binding assays and, more importantly, exclude any MC-R–mediated signaling of α-MSH.

**FIGURE 4.** KoPT does not act via α-MSH binding sites, does not bind to MC-1R, and has no melanotrophic activity. For blocking experiments, SZ95 sebocytes were stimulated with IL-1β (1 ng/ml), KoPT (10⁻⁶ M) in the presence or absence of a synthetic ASIP peptide (10⁻⁶ M) for 8 h. Relative mRNA amounts of IL-6 (A) and IL-8 (B) were determined by real-time RT-PCR. *p < 0.01 versus IL-1β–treated cells. Competition binding studies with SZ95 sebocytes and mouse B16-F1 melanoma cells, using ¹²⁵I-NDP–α-MSH radioligand and KoPT (10⁻³–10⁻⁸ or 10⁻¹⁰ M, respectively) or α-MSH (10⁻⁶–10⁻¹¹ or 10⁻¹² M, respectively) as competitors, are shown in (C). The incubation conditions were the same for both B16-F1 and SZ95 cells (15˚C/3 h); the data were identical when SZ95 cells were incubated at 37˚C/2 h (data not shown). Depicted is one representative experiment of 3 independent experiments with similar results. In each individual experiment, different treatments were determined by six individual determinations. Depicted data are means ± SD from one experiment. δ₅₀ binding of the radioligand in the absence of competitor peptides. For melanin assays (D), B16-F1 melanoma cells were cultured for 72 h with KoPT at doses indicated or with NDP-α–MSH (10⁻⁸ M) as positive control. *p < 0.001 versus control.
KnPT as an essential component of its biological effect. In accordance with the latter finding, KnPT—in contrast to α-MSH—failed to bind to B16-F1 murine melanoma cells, too (Fig. 4C). To finally complement the data of these binding assays with a more functional test, melanin production was determined in B16-F1 melanoma cells in response to KnPT and NDP-α-MSH. NDP-α-MSH (10^-8 M) significantly increased the secreted amount of melanin into the culture supernatants of B16-F1 melanoma cells after 72 h of incubation, whereas KnPT at doses from 10^-6 to 10^-10 M did not elicit any melanotropic effect (Fig. 4D).

These findings demonstrate that KnPT acts independently of MC-Rs. Moreover, lack of a pigment-inducing effect represents important preclinical information for the therapeutic exploitation of this anti-inflammatory tripeptide.

KnPT reduces IL-1β–fluorochrome cell surface binding in SZ95 sebocytes

Because the amino acid sequence of KPT is contained within the IL-1β (fragment 193–195), we next considered the possibility of KnPT binding to the signaling-sufficient IL-1RI. RT-PCR analysis disclosed the expression of IL-1RI in SZ95 sebocytes (Fig. 5A). However, subsequent radioligand binding studies using iodinated IL-1β did not yield specific binding in SZ95 sebocytes, possibly owing to changes in the binding behavior of the radiotracer by the incorporated [125I] (data not shown). Thus, we performed an alternative approach in which human IL-1β was fluorochrome-labeled in vitro with Alexa Fluor 488. SZ95 sebocytes were then incubated with the IL-1β–fluorochrome. Treatment of cells with the fluorochrome-tagged IL-1β resulted in surface labeling, which was reduced in the presence of non–fluorochrome-labeled IL-1β (1 ng/ml; reduction of fluorescence intensity versus control: -32.0 ± 14.4%). KnPT (10^-7 M) similarly reduced cell surface binding of the fluorochrome-tagged IL-1β (reduction of fluorescence intensity versus control: -45.3 ± 13.3%) (Fig. 5B).

Computer modeling suggests binding of KnPT to the IL-1RI antagonizing binding of IL-1β

To gain a better understanding, we used computer modeling for a closer look into a possible binding of KnPT to IL-1RI. The crystal structures of the IL-1β/IL-1RI complex revealed that the amino acids in positions 193–195 of IL-1β do not interact with the receptor (Fig. 6A). We thus turned to previously reported site-directed mutation studies to investigate if any Lys, Pro, or Thr

![FIGURE 5.](http://example.com/figure5.png)

**FIGURE 5.** Expression of IL-1RI in SZ95 sebocytes and reduced fluorochrome–IL-1β cell surface binding by KnPT. A. Expression of IL-1RI in SZ95 sebocytes, as shown by RT-PCR. NC1, negative control using H2O as template; NC2, contamination control RNA without reverse transcription as template. B, SZ95 sebocytes were labeled with Alexa Fluor 488–IL-1β for 15 min on ice. Specificity of the binding was demonstrated by preincubation with 1 ng/ml of nonlabeled IL-1β. KnPT (10^-7 M) similarly reduced cell surface binding of the fluorochrome-tagged IL-1β. Depicted is one of two independent experiments with similar results.

![FIGURE 6.](http://example.com/figure6.png)

**FIGURE 6.** Computer modeling of predicted complexes between IL-1β and IL-1RI versus KnPT and IL-1RI. A. Analysis of the solved crystal structure of the IL-1R/IL-1β complex shows that the equivalent region of IL-1β (shown in red; residues 193–195) corresponding to KPT does not directly bind to the Ig-like C2 domain of IL-1R (C1 domain, blue; C2 domain 2, yellow; C3 domain 3, purple). Inset shows residues 240–264 of the third Ig-C2-like domain, where KnPT binds, for comparison. B, KnPT (green) antagonizes IL-1β by binding to the third Ig-like C2 domain of the IL-1R (residues 209–311), binding between a loop of two β strands (residues 240–264). Lys1 is the most important residue, forming H-bonds with Glu252 and Asp253 with its amine group and also with Glu252 and Asp251 with the N-terminal side chain group. Thr3 forms H-bonds to Asn246 and to Ser248, whereas the reasonably close proximity of D-Pro2 to Ile249 may indicate hydrophobic interactions. Residues of IL-1R binding to KnPT are shown in normal atomic coloring (oxygen, red; carbon, cyan; nitrogen, blue; hydrogen, white).
residues on IL-1β had been shown to be important or critical for receptor binding. Interestingly, Lys209 of IL-1β was found to be absolutely essential for activity, with mutants showing <1% of activity (37, 38). Examination of the crystal structure of the IL-1β/IL-1RI complex showed that the residue Lys209 interacted with Glu252, which is found on a loop between two β strands (fragment 240–264) with the sequence IAYWKWNGSVIDE252. DDPVLGEDYYSV264, which is itself located on the third Ig-like domain of IL-1RI. This loop on the receptor is rich in acidic residues; in particular, there are 3 Asp and 1 Glu residues in a continuous sequence (residues 251–254). Therefore, models were constructed on the basis that Lys1 in KoPT might enable strong binding via interaction with one or more of these acidic residues. In addition, the possibility was investigated that Thr3 in KoPT might also interact with residues on the above loop. In the final model, a complex between KoPT and IL-1RI was predicted in which the e-amino group of Lys1 side chain H-bonds to Glu252 and Asp253, whereas the N-terminal amino group of Lys1 binds also Glu252 and to Asp251. The C-terminal carboxylic acid group of Thr3 binds to Ass246 and Ser248. d-Pro2 may form hydrophobic interactions with Val249 (Fig. 6B).

Discussion

In this report, we have investigated anti-inflammatory potential and its underlying molecular mechanism in SZ95 sebocytes of KoPT, a tripeptide derivative of the C-terminal end of α-MSH. Our findings provide evidence for a significant suppressive effect by this small molecule in a wide range of concentrations (micromolar to subnanomolar) on IL-1β-induced IL-6 and IL-8 expression in the above in vitro cell culture model. Because there is no established animal model for acne, it is of some interest that KoPT, in fact, possesses anti-inflammatory effects in vivo [e.g., in mouse models of experimentally induced colitis (39)]. The latter finding may indicate that KoPT may possess anti-inflammatory effector mechanisms additional to antagonism of IL-1β at the IL-1R level. Interestingly, the KoPT-related α-MSH tripeptide homolog KPV was reported to exert its anti-inflammatory effects via PepT1, an H+-coupled oligopeptide transporter expressed in the gut (40). Thus, we cannot rule out that peptide transporters such as PepT1 may also be involved in the anti-inflammatory effect of KoPT.

As demonstrated by our mechanistic studies, KoPT attenuates the activation of NF-κB pathway, presumably by binding to IL-1RI and consequently by reduction of IL-1β-mediated intracellular ROS production. ROS formation is a well-known upstream signal mediator activating the redox-sensitive transcription factor NF-κB (33). The role of intracellular ROS as a mediator of IL-1β at the IL-1R1 level.

In summary, we have shown for the first time that KoPT has potent anti-inflammatory effects under in vitro conditions. The peptide acts antagonistically to IL-1β, presumably by binding to the signaling-sufficient IL-1RI. Consequently, IL-1β-mediated intracellular ROS formation, NF-κB activation, and IL-6 and IL-8 expression are attenuated. The lack of melanotropic activity suggests that this peptide is a promising future candidate for the treatment of many inflammatory human diseases, not only those involving inflamed sebaceous glands.

Acknowledgments

We thank Britta Ringelkamp, Mara Apel, and Heidi Tanner for expert technical assistance and Claus Kerkhoff for critical reading of the manuscript.

Disclosures

M.B. is an inventor of antioxidative tripeptides (EP070222401). C.C.Z. is an inventor of the SZ95 sebaceous gland cell line (AU770518B, CA2360762, CN100366735C, DEI9903920, EP1151082, HU0200048, IL144683, JP2000-597413, KR689120, PL194865, US2002034820). All other authors have no financial conflicts of interest.

References

8. Nakatsuji, T., Y. T. Liu, C. P. Huang, C. C. Zouboulis, R. L. Gallo, and C. M. Huang. 2008. Antibodies elicited by inactivated propionibacterium acnes-based vaccines exert protective immunity and attenuate the IL-8 production in

Downloaded from http://www.jimmunol.org/ by guest on August 1, 2022


<table>
<thead>
<tr>
<th>Ctr.</th>
<th>10⁻⁶ M</th>
<th>10⁻⁸ M</th>
<th>10⁻¹⁰ M</th>
</tr>
</thead>
<tbody>
<tr>
<td>KDPT Rel. mRNA expression (fold change)</td>
<td>IL-6</td>
<td>IL-8</td>
<td></td>
</tr>
</tbody>
</table>