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Peptide LKEKK Reduces Acute and Chronic Skin Inflammation

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ABSTRACT

We have established that the peptide LKEKK (Np5) corresponding to the sequence 16-20 of thymosin-α1 and to the sequence 131-135 of interferon-α2, in the concentration range 50 300 μg/ear reduces in a dose-dependent manner phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced skin edema in mice .Tested in parallel peptide with inverted sequence (iNp5, KKEKL, 150-300 μg/ear) was inactive, indicating high specificity of the Np5 action. In the concentration range of 5 20 μM Np5 significantly decrease the TNF-α-induced production by normal human keranocytes of pro-inflammatory mediators IL-6 and IL-1β. Thus, Np5t has a pronounced anti-inflammatory activity in vivo and in vitro.

KEYWORDS: Peptide; Receptor; Cytokine; Keratinocyte; Skin Inflammation

INTRODUCTION

Several years ago we synthesized peptide LKEKK (Np5) corresponding to the sequence 16-20 of thymosin-α1 and the sequence 131-135 of interferon-α2, and found that it significantly reduces TNF-α-stimulated pro-inflammatory cytokine (IL-6, IL-8, and IL-1β) expression and increases the expression of the anti-inflammatory cytokine IL-10 in human Caco-2 intestinal epithelial cells via the soluble guanylate cyclase-dependent signal pathway (Navolotskaya et al., 2019a). Moreover, in a mouse model of dextran sodium sulfate-induced colitis the peptide (20 mg/kg body weight orally for 14 days) decreased the production of TNF-α and IL-6, as well as the severity of inflammation. Thus, Np5 is able to suppress inflammation in vitro and in vivo.

Recently we prepared tritium labeled Np5 and established that it binds with high affinity and specificity to normal human epidermal keratinocytes (NHEK) (Navolotskaya et al., 2020). Furthermore, it was shown that in the concentration range of 50 1000 nM Np5 increased in a dose-dependent manner the soluble guanylate cyclase activity in NHEK, significantly reduced IL-17A-induced secretion of pro-inflammatory cytokines (TNF-α, IL-6, and IL-1α) and increased the production of the anti-inflammatory cytokine IL-10 by the

cells in vitro. Thus, it was shown that the action of the peptide is mediated through a soluble guanylate cyclase-dependent signaling pathway.

The aim of this work is to study the activity of Np5 in models of acute and chronic cutaneous inflammation in mice.

MATERIALS AND METHODS

Animals: BALB/c mice (8–10 weeks old)) were obtained from the nursery of the Branch of Institute of Bioorganic Chemistry. The animals were housed 6 per cage under standard laboratory conditions 12/12 h light-dark cycle (lights on at 7.00 a.m.) at an ambient temperature 20-22 °C. The experiments procedures followed the guidelines for the care and use of laboratory animals, and they were approved by the Institutional Ethics Committee.

Chemicals: Human Keratinocyte Medium EpiGro was obtained from Cell Applications, Inc. (USA), IL-17A, TNF-a, IL-1a, and other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Peptides: Human thymosin-α1 and human interferon-α2 were obtained from Immundiagnostik AG (Germany). Peptides LKEKK (Np5) and KKEKL (iNp5) were synthesized on an Applied Biosystems Model 430A automatic synthesizer (USA) using the Boc/Bzl tactics of peptide chain elongation as described previously (Schnolzer et al., 1992). The peptides were purified to homogeneous state by preparative reverse-phase HPLC (Gilson chromatograph, France) on a Delta Pack C18 column, 100A (39×150 mm, mesh size 5 □m; flow rate 10 mL/min, elution with 0.1% TFA, gradient of acetonitrile 10–40% in 30 min). The molecular masses of peptides were determined by fast atom bombardment mass spectrometric analysis (Finnigan mass spectrometer, San Jose, CA). The data of amino acid analysis (hydrolysis by 6 M HCl, 22 h, 110 °C; LKB 4151 Alpha Plus amino acid analyzer, Sweden) and mass spectrum analysis are presented in (Table 1).

Table 1: Main characteristics of the peptides

Peptide	Purity, %	Amino acid analysis data	Molecular mass, D
Np5	>97	Glu 1.07, Leu 1.02, Lys 3.35	645.7 (calculated value - 644.87)
iNp5	>97	Glu 1.13, Leu 1.04, Lys 3.31	648.6 (644.87)

Keratinocyte Cultures: Normal human epidermal keratinocytes (NHEK) were obtained from Cell Applications, Inc. (USA) and were cultured for 24 h in Keratinocyte serum-free medium EpiGro containing EpiLife undefined growth supplement (Thermo Fisher Scientific, USA) in a 5% CO2 incubator at 37°C and were used at the second or third passage. Cells were pretreated with Np5 or iNp5 (10–1000 nM) for 1 h before stimulation with recombinant human IL-17A (20 ng/mL, for 24 h). (Chorachoo et al., 2018).

Model of Acute Inflammation in Mice: The inner and outer surfaces of the right ear of mice (10 animals per group) were treated with Np5 or iNp5 (10, 50, 100, 150 and 300 μg dissolved in 20 μl of base medium (2% dimethyl sulfoxide (DMSO) + 20% propylene glycol + 70% acetone, three times with an interval of 15 minutes). A group of mice treated with base medium according to the same scheme served as a negative control. Animals of the positive control group were treated with dexamethasone (0.05 mg/ear in acetone). After 15 minutes, 20 μL of TPA (12-O-tetradecanoylphorbol-13-acetate, 2.0 μg/ear in acetone) was applied to the right ear of mice. Edema (increase in ear thickness) was measured with a digital thickness meter (Mitutoyo Corporation, Japan) before and 5 hours after TPA application. The ear biopsy samples were collected and stored at 80 °C.

Model of Chronic Inflammatory in Mice: Effect of Np5 or iNp5 on TPA-induced chronic skin inflammation was examined according to the previously described procedure (Stanley et al., 1991): 20 μL of TPA solution (2.0 μg/ear in acetone), or acetone (vehicle) was applied with a micropipette to the inner and outer surfaces of the right ear 6 times every other day. On days 7–9, the mice were treated on the inner and outer surfaces of the right ear with Np5 (100, 150 and 300 μg/ear), or dexamethasone (0.05 mg/ear in acetone, positive control) twice a day. Ear thickness was measured using a digital thickness gauge at 5 hours after the last TPA treatment. The ear biopsy samples were collected and stored at −80 °C.

Analysis of Cytokine Production in Inflammatory Regions: Ear biopsy samples (n = 9) were weighed and homogenized in 1 mL of T-PER tissue protein extraction reagent (Thermo Scientific) containing a cocktail of protease inhibitors and incubated on ice for 30 min. Homogenates were centrifuged at 12000×g for 20 min at 4°C. After centrifugation, TNF-α, IL-6 and IL-1β in the supernatants were measured by ELISA. Results were expressed as cytokine amount (pg) per 1 mg total protein. ELISAs were carried out according to the manufacturer's instructions (BD Biosciences, San Jose, CA). The protein concentration was determined by the Lowry method (Lowry et al., 1956) using bovine serum albumin as a standard. Data are presented as mean ± SEM.

Measurement of TNF-α-induced production of IL-6 and IL-1β in keratinocytes: Human keratinocytes were seeded in triplicate into a 24-well plate (1×105 per well). When the cell confluence in each well reached about 60%, the culture supernatants were removed and the fresh medium was added. The cells were then treat-

ed with TNF-α (10 ng/mL) in the presence of 0.5–20 μM Np5 for 24 h at 37°C. Culture supernatants were analyzed using IL-6 and IL-1β ELISA kits (BD Biosciences, San Jose, CA) according to the manufacturer's instructions.

Histology: Ear biopsies were fixed in 10% neutral buffered formalin, embedded in paraffin, cut into 5 μm sections, and stained with hematoxylin and eosin. Leukocyte accumulation and edema were evaluated using a magnification of 200×.

Statistical Analysis: Data are expressed as means ± SEM. Student's t-test was used when comparisons were made only between the two groups. Differences were considered significant when $p < 0.05$.

RESULTS

The main characteristics of the synthesized peptides (purity, amino acid content, and molecular mass) are shown in (Table 1).

Effect of Np5 on Skin Edema in TPA-induced Edema Model: We evaluated the anti-inflammatory activity of the peptide in a mouse model of TPA-induced acute contact dermatitis. Increased skin thickening is the first sign of skin irritation and local inflammation, which occurs due to increased vascular permeability, dermal edema and proliferation of epidermal keratinocytes. Results presented in Table 1 show that local exposure to TPA results in a significant increase in ear thickness (Table 1). Topical application of base medium (2% DMSO + 20% propylene glycol + 70% acetone, negative control) alone did not lead to a noticeable change in skin thickness. At the same time Np5 (50 300 μg/ear) or positive control (dexamethasone 0.05 mg/ear) significantly reduced the increase in ear thickening. Peptide iNp5 with inverted sequence (150-300 μg/ear) tested in parallel was inactive.

Histological studies of hematoxylin and eosin (H&E) -stained ear tissue sections from TPA-treated mice showed that TPA-treatment resulted in a marked increase in ear thickness with clear evidence of edema and the appearance of a significant number of inflammatory cells in the dermis (Figure 1A, B). Topical application of Np5 (300 μg/ear) significantly reduced ear thickness and associated pathologic indicators. These results provide additional evidence that Np5 reduces TPA-induced acute contact dermatitis (Table 2).

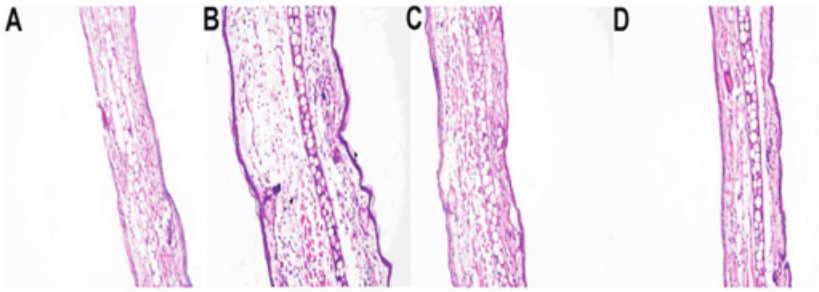

Figure 1: Histological presentation of H&E-stained sections of mice ears sensitized with topical application of acetone (A) or TPA (B - D). Representative sections from the following groups are shown: 5 h post-treatment with the vehicle (B), Np5 (300 μg/ear) (C) or DEX (0.05 mg/ear) (D), (200 × magnification).

Table 2: Effect of Np5 and iNp5 on TPA-induced changes in mouse ear thickness

Treatment	Increase in ear thickness (μm)
Base medium (negative control)	< 10
Dexamethasone (0.05 mg/ear in acetone, positive control)	60 ± 3*
TPA (12-O-tetradecanoylphorbol-13-acetate solution (2.0 μg/ear in acetone)	320 ± 24
Np5 (10 μg/ear in base medium)	280 ± 27
Np5 (50 μg/ear in base medium)	200 ± 16*
Np5 (100 μg/ear in base medium)	160 ± 12*
Np5 (150 μg/ear in base medium)	140 ± 13*
Np5 (300 μg/ear in base medium)	140 ± 10*
rNp5 (150 μg/ear in base medium)	325 ± 28
rNp5 (300 μg/ear in base medium)	330 ± 26

Note: ear thickness was measured at 5 h after TPA treatment. The data were expressed as mean ± S.E.M. (n=10).
* $p<0.05$ compared with TPA-treatment.

Influence of Np5 On the Pro-Inflammatory Cytokines Expression in Areas of Inflammation

It has previously been shown that topical exposure to TPA results in a marked increase in the pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β) levels in mouse ear biopsy homogenates (De Vry et al, 2005; Murakawa et al., 2006). The results shown in Table 3 indicate that TNF-α, IL-6 and IL-1β levels increased dramatically in TPA-treated ears. At the same time, treatment with TPA plus Np5 (50-300 µg/ear) significantly reduced the levels of these cytokines in a dose-dependent manner. It should be noted that the effectiveness of the peptide at concentrations of 150-300 µg/ear was comparable to that of DEX. These data indicate that Np5 can reduce skin inflammation by inhibiting the production of TNF-α, IL-6 and IL-1β (Table 3).

Effect of Np5 On TPA-Induced Chronic Inflammation

To conclusively conclude that Np5 has anti-inflammatory activity in vivo, we investigated its action in a model of chronic inflammation of the skin induced by multiple applications of TPA to the ears of mice. The inflammatory response, characterized by an increase in ear weight, infiltration of inflammatory cells, and epidermal hyperplasia persists in this model [7]. The results in Table 4 show that exposure to TPA results in a significant increase in both the thickness and weight of the ears. It can be seen that Np5 (100 300 µg/ear) markedly inhibits the increase in ear thickness and weight. It should be noted that the peptide activity (150 300 µg/ear) was comparable to the DEX activity.

Examination of H&E-stained ear sections from multiple TPA-treated mice showed that multiple TPA application resulted in a marked increase in ear thickness, and epidermal hyperplasia. (Figure 2A, B). Treatment with Np5 (300 µg/ear) significantly reduced ear thickness and associated pathologic signs (Figure 2C, D). These data support the ability of the peptide to suppress persistent inflammatory lesion with multiple topical applications (Table 4).

Table 3: Effect of Np5 on TPA-induced expression of TNF-α, IL-6, and IL-1β levels in mouse ear biopsy homogenates

Treatment	Cytokine levels (pg/mg total protein)		
	TNF-α	IL-6	IL-1β
Base medium (negative control)	122 ± 13*	257 ± 23*	317 ± 33*
TPA (12-O-tetradecanoylphorbol-13-acetate solution (2.0 µg/ear in acetone)	285 ± 24	878 ± 75	794 ± 72*
Dexamethasone (0.05 mg/ear in acetone, positive control)	166 ± 15*	365 ± 33*	366 ± 39*
Np5 (50 µg/ear in base medium)	244 ± 27	794 ± 69	665 ± 73
Np5 (100 µg/ear in base medium)	193 ± 17*	426 ± 38*	389 ± 29*
Np5 (150 µg/ear in base medium)	175 ± 18*	387 ± 35*	378 ± 35*
Np5 (300 µg/ear in base medium)	172 ± 19*	379 ± 37**	272 ± 38*

Note: Ear punch biopsies were taken at 5 h after TPA application and tissue homogenates were examined for cytokine production by ELISA. The data were expressed as mean ± S.E.M. (n=9). * p<0.05 compared with TPA-treatment.

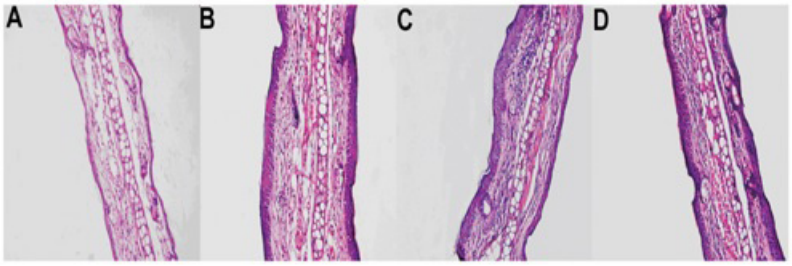

Figure 2: Histological presentation of H&E-stained sections of mice ears sensitized with multiple topical application of TPA. A chronic irritant contact dermatitis is evolved by the TPA application every other day for 9 d, and ears were harvested at 5 h after the last acetone (A), TPA (B), Np5 (300 µg/ear) (C), or DEX (0.05 mg/ear) (D) treatment. Representative sections from each group are shown (200× magnifications).

Table 4: Effect of Np5 on TPA multiple application induced changes in murine ear thickness and weight

Treatment	Increase in ear thickness (µm)	Increase in ear weight (mg)
Base medium (negative control)	< 10	-
Dexamethasone (0.05 mg/ear in acetone, positive control)	80 ± 5*	4.2 ± 0.3*
TPA (12-O-tetradecanoylphorbol-13-acetate solution (2.0 µg/ear in acetone)	360 ± 32	7.9 ± 0.5
Np5 (50 µg/ear in base medium)	260 ± 17*	6.3 ± 0.4*
Np5 (100 µg/ear in base medium)	180 ± 12*	5.2 ± 0.5*
Np5 (150 µg/ear in base medium)	110 ± 8*	4.8 ± 0.6*
Np5 (300 µg/ear in base medium)	115 ± 10*	4.6 ± 0.5*

Note: ear thickness was measured at 5 h after TPA treatment. The data were expressed as mean ± S.E.M. (n=10). * p<0.05 compared with TPA-treatment.

Effect of Np5 On Pro-Inflammatory Cytokines in Human Keratinocytes

As shown in Table 5. Np5 in the concentration range of 5 20 μM significantly reduced the TNF-α-induced IL-6 and IL-1β production in a dose-dependent manner. These results indicated that the peptide is able to inhibit the production of these pro-inflammatory cytokines in human keratinocytes (Table 5).

Table 5: Effect of Np5 on the production of pro-inflammatory mediators in human keratinocytes

Treatment	IL-1β (pg/mL)	IL-6 (pg/mL)
Base medium (negative control)	16 ± 3	4 ± 1
TNF-α (10 ng/mL)	112 ± 13	87 ± 9
TNF-α (10 ng/mL) in the presence of Np5 (0.5 μM)	106 ± 7	84 ± 9
TNF-α (10 ng/mL) in the presence of Np5 (1 μM)	91 ± 10	77 ± 8
TNF-α (10 ng/mL) in the presence of Np5 (5 μM)	84 ± 8*	68 ± 7*
TNF-α (10 ng/mL) in the presence of Np5 (10 μM)	78 ± 6*	60 ± 8*
TNF-α (10 ng/mL) in the presence of Np5 (10 μM)	50 ± 7*	39 ± 8*
TNF-α (10 ng/mL) in the presence of Np5 (15 μM)	34 ± 5*	28 ± 4*
TNF-α (10 ng/mL) in the presence of Np5 (20 μM)	35 ± 4*	25 ± 3*

Note: the concentration of IL-6 and IL-1β in the culture supernatant was determined by ELISA. The data were expressed as mean ±S.E.M. of triplicate cultures, * $p<0.05$ compared with the group in which keratinocytes were activated with TNF-α.

DISCUSSION

Several years ago we synthesized the peptide LKEKK (Np5) corresponding to the sequence 16-20 of thymosin-α1 and the sequence 131-135 of interferon-α2, and showed that it is able to bind with high affinity and specificity human T and B lymphocytes (Navolotskaya et al., 2016a, 2017a), rat intestinal epithelial cell membranes (Navolotskaya et al. 2016b, 2017b), rat IEC-6 (Navolotskaya et al., 2018a, 2018b), and human Caco-2 (Navolotskaya et al., 2018b) intestinal epithelial cells, murine Raw 264.7 macrophage-like cells (Navolotskaya et al., 2019a). In all of the above cases, treatment of cells or membranes with proteases did not affect the binding, suggesting the non-protein nature of the peptide binding site. At the same time, the Np5 specific binding was competitively inhibited by TM- α1, IFN-α2, and cholera toxin B subunit. It was suggested that the peptide can bind to the toxin receptor, which is known to be GM1-glanglioside (Cuatrecasas, 1973; Holmgren et al., 1973).

Recently we demonstrated the anti-inflammatory activity of Np5 in a model of TNF-induced inflammation in human Caco-2 intestinal epithelial cells in vitro and in a mouse model of dextran sodium sulfate-induced colitis in vivo (Navolotskaya et al., 2019b). Then we examined the anti-inflammatory potential of Np5 using an in vitro model of IL-17A-induced inflammation in normal human keratinocytes (Navolotskaya et al., 2020). For this, cells were treated with the peptide in the concentration range of 10 1000 nM, and IL-17A (20 ng/mL) was added to induce inflammation. In parallel, the peptide with inverted amino acid sequence iNp5 was tested as a negative control. Our experiments showed that pre-treatment of keratinocytes with Np5 at the concentrations of 50 1000 nM significantly reduced in a dose-dependent manner the IL-17A-induced secretion of three major pro-inflammatory cytokines (TNF-α, IL-6, and IL-1α), and at the same time, enhanced the production of anti-inflammatory Il-10. The peptide with inverted amino acid sequence iNp5 was inactive; which indicates a high specificity of the Np5 action.

We found that the effects of Np5 on various types of cells (human T and B lymphocytes, human Caco-2 and rat IEC-6 intestinal epithelial cells, murine Raw 264.7 macrophage-like cells) are mediated by a cGMP-dependent pathway (Navolotskaya et al. 2017a, 2018b). Moreover, we have shown that Np5 binds with high affinity to human keratinocytes (Kd 2.6 nM) and increases in a dose-dependent manner the sGC activity (Navolotskaya et al., 2020). We here investigated the effect of the peptide on the ability of keratinocytes to IL-17A-induced secretion of TNF-α and IL-1α under partial or total absence of the sGC activity. Inhibition the enzyme activity is achieved using an inhibitor of sGC ODQ which oxidizes the haem prosthetic group to which NO binds (Feelisch et al., 1999). It was found that the decrease in enzyme activity was accompanied by a loss of the ability of the peptide to inhibit the cytokine secretion. Therefore, Np5 reduces the pro-inflammatory cytokine secretion in IL-17A-stimulated human keratinocytes via sGC.

In the present study, unconditional evidence was obtained for the ability of Np5 to suppress the inflammatory process: the peptide reduced in a dose-dependent manner TPA-induced skin edema in mice in vivo, and it significantly decreased the TNF-α-induced production by normal human keranocytes of pro-inflammatory mediators IL-6 and IL-1β in vitro.

CONCLUSION

Np5 (LKEKK), a short peptide with a simple structure, exhibiting anti-inflammatory effects in vitro and in vivo, has significant potential as an anti-inflammatory drug.

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