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Research Article

Anti-inflammatory Effect of Peptide LKEKK on Keratinocytes

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Abstract

We synthesized the peptide LKEKK (Np5) corresponding to the sequence 16-20 of thymosin-α1 and to the sequence 131-135 of interferon-α2, prepared [³H]Np5 and found that it binds with high affinity and specificity to human keratinocytes. In the concentration range of 50 1000 nM Np5 increased in a dose-dependent manner the soluble guanylate cyclase activity in keratinocytes, 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*. It was shown that the action of the peptide is mediated through a soluble guanylate cyclase-dependent signaling pathway.

Keywords: Protein; Peptide; Receptor; Cytokine; Keratinocyte; Inflammation; Skin

Introduction

The skin is the largest organ of the mammals with three main functions: protection, regulation and sensation. Mammalian skin is composed of two primary layers: the epidermis, which forms a protective barrier on the surface of the body, and the dermis, which serves as a location for the appendages of skin. The epidermis is a stratified squamous epithelium consisting of proliferating basal and differentiated suprabasal keratinocytes that are the major cells of the epidermis. Keratinocytes in the basal layer of the epidermis proliferate, and the daughter cells move up and undergo multiple stages of cell differentiation, in order to eventually become nucleated. Keratinocytes respond to various environmental factors and play a crucial role in the regulation of skin inflammation. Five proinflammatory cytokines produced by keratinocytes have been shown to be involved in the induction of the skin inflammation: IL-17A, IL-22, oncostatin M, TNF-α, and IL-1α [1]. The role of individual cytokines in the development of the inflammatory response of the skin has been established: IL-22 and oncostatin M control epidermal hyperplasia and loss of differentiation, while IL-1α, IL-17A, and TNF-α provide the activation of innate immunity [2].

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, capable of specifically binding to human T and B lymphocytes [3,4], rat intestinal epithelial cell membranes [5,6], rat IEC-6 [7,8] and human Caco-2 [8] intestinal epithelial cells, murine Raw 264.7 macrophage-like cells [9]. In all cases treatment with proteases did not affect the binding, suggesting the non-protein nature of the peptide receptor. The Np5 binding was competitively inhibited by TM- α1, IFN-α2, and cholera toxin B subunit. We suggested that this receptor could be the toxin receptor, which is known to be a GM1-glanglioside [10,11].

Recently we found that Np5 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 [12]. 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*. The purpose of this study is to characterize the effect of Np5 on normal human keratinocytes.

Materials and Methods

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 (St. Louis, MO).

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. 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 µm; 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, 22h, 110°C; LKB 4151 Alpha Plus amino acid analyzer, Sweden) and mass spectrum analysis are presented in Table 1.

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Peptide	Purity, %	Amino acid analysis data	Molecular mass, D
Np5	>98	Glu 1.08, Leu 1.00, Lys 3.32	645.4 (calculated value - 644.87)
iNp5	>97	Glu 1.14, Leu 1.05, Lys 3.30	648.5 (644.87)

Table 1: Main characteristics of the peptides.

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% CO_2 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) [13]. 4.4. Preparation of [³H]Np5.

[³H]Np5 was obtained by the reaction of high-temperature .solid-phase catalytic isotope exchange [14]. Aluminum oxide (50 g) was added to a solution of immunorphin (2 mg) or octarphin (2 mg) in water (0.5 mL), and the solution was evaporated on a rotor evaporator. Aluminum oxide with the peptide applied was mixed with 10 mg of catalyst (5% Rh/Al_2O_3). The solid mixture obtained was placed in a 10-mL ampoule. The ampoule was evacuated, filled with gaseous tritium to a pressure of 250 torr, heated to 170°C, and kept at this temperature for 20 min. The ampoule was then cooled, vacuumized, blown with hydrogen, and vacuumized again. The labeled peptide was extracted from the solid reaction mixture by two portions of 50% aqueous ethanol (3 mL each), and the combined solution was evaporated. Labile tritium was removed be repeating the procedure twice. [³H]Np5 was purified by HPLC with a Beckman spectrophotometer at 254 and 280 nm on a column of Kromasil (4×150 mm; the granulation was 5 μm, 20°C). The elution was with 0.1% TFA using a gradient of methanol gradient 42-70% in 20 min; the flow rate was 3 mL/min. The incorporation of tritium into the peptide was calculated by liquid scintillation counting.

Binding assay

The binding of [³H]Np5 to the cells was assayed in 1 mL of Keratinocyte serum-free medium EpiGro, containing 10 mM Hepes, 20 mM NaN_3 and 0.6 mg/mL PMSF (pH 7.4): 100 μl labeled peptde (concentration range 10^{-10}-10^{-7} M, each concentration point in triplicate) plus 100 μl medium (for total binding) or 10^{-4} M unlabeled peptide (for nonspecific binding) were added to 800 μl cell suspension (10^6 cells) and incubated at 4°C for 30 min. Then the samples were filtered through Whatman GF/A glass fiber filters to separate cell-bound labeled protein from non-bound (free) one. Filters were washed three times with 5 mL ice-cold saline. Radioactivity was counted using Mini-Gamma counter (LKB, Sweden). The specific binding of labeled peptide to cells was determined as the difference between total and nonspecific binding that was measured in the presence of 10^{-4} M unlabeled peptide. The specific binding of [³H]Np5 to the cells was further characterized by the equilibrium dissociation constant K_d, To determine K_d, the ratio between molar concentrations of the bound (B) and free (F) labeled protein was plotted against molar concentration of the bound labeled protein (B) [15].

Competition assay

To estimate the inhibitory effects of TM-α_1, IFN-α_2 and iNp5, the cells (10^6/mL) were incubated with 5 nM labeled peptide and one of the tested ligands (concentration range, 10^{-12}-10^{-5} M; three measurements for each concentration) as described above. The inhibition constant (K_i) was calculated using the formula: $K_i = [IC_{50}]/(1 + [L]/K_d)$ [16], where [L] is the labeled peptide molar concentration; K_d is the equilibrium dissociation constant of the labeled peptide–receptor complex; IC_{50} is the concentration of unlabeled ligand causing 50% inhibition of the labeled peptide specific binding. IC_{50} was determined graphically from the inhibition plots. The value of K_d was determined as described above. The data are presented as the means ± SEM of at least three independent experiments.

Measurement of soluble (sGC) and particulate (pGC) guanylate cyclase activity

Subcellular fractions from human keratinocytes were obtained at 4°C as described earlier [8]. The guanylate cyclase (sGC and pGC) activity was measured by monitoring the conversion of [α-³²P]GTP to [³²P]cGMP [17]; the product was isolated by precipitation with zinc carbonate and chromatography on a column of aluminum oxide [18]. The enzyme activity was expressed as the amount of cGMP produced in 10 min (in nanomoles per 1 mg protein). The protein concentration was determined by the Lowry method using bovine serum albumin as a standard. To inhibit the activity of sGC, an inhibitor of the enzyme ODQ (1H-[1,2,4]oxadiazolo[4,3-α]quinoxalin-1-one in the concentration range of 5-100 μM was used [19].

Cytokine ELISAs

To measure the concentrations of cytokines in keratinocytes, cells were homogenized in three volumes of ice-cold PBS containing 1 mM PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 10 μg/mL pepstatin A (Sigma-Aldrich) using a POLYTRON® PT 1200 E (Kinematica AG., Switzerland) and centrifuged at 12,000 ×g for 10 min at 4°C. The protein concentration was determined by the Lowry method [20] using bovine serum albumin as a standard. was measured by ELISA. Results are expressed as cytokine amount per total protein concentration. ELISAs were carried out using according to the manufacturer's instructions (BD Biosciences, San Jose, CA). Data are presented as mean ± SEM (Figure 1).

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. The HSCIE reaction with subsequent peptide purification yielded [³H]Np5 with specific activity of 28 Ci/mmol. The retention time for both labeled and unlabeled peptides on a Kromasil C18 column (see "Materials and methods") was 11 min; the 254/280 nm absorbance ratio for the labeled and unlabeled peptides was the same, thereby confirming that hydrogen substitution with tritium did not affect chemical structure of the peptide.

Binding of [³H]Np5 to human keratinocytes

The experiments showed that [³H]Np5 binds specifically to the cells, and this binding is reversible and saturable; dynamic equilibrium in the system labeled peptide-receptor was established approximately after 30 min and remained in this state for at least 1 h.
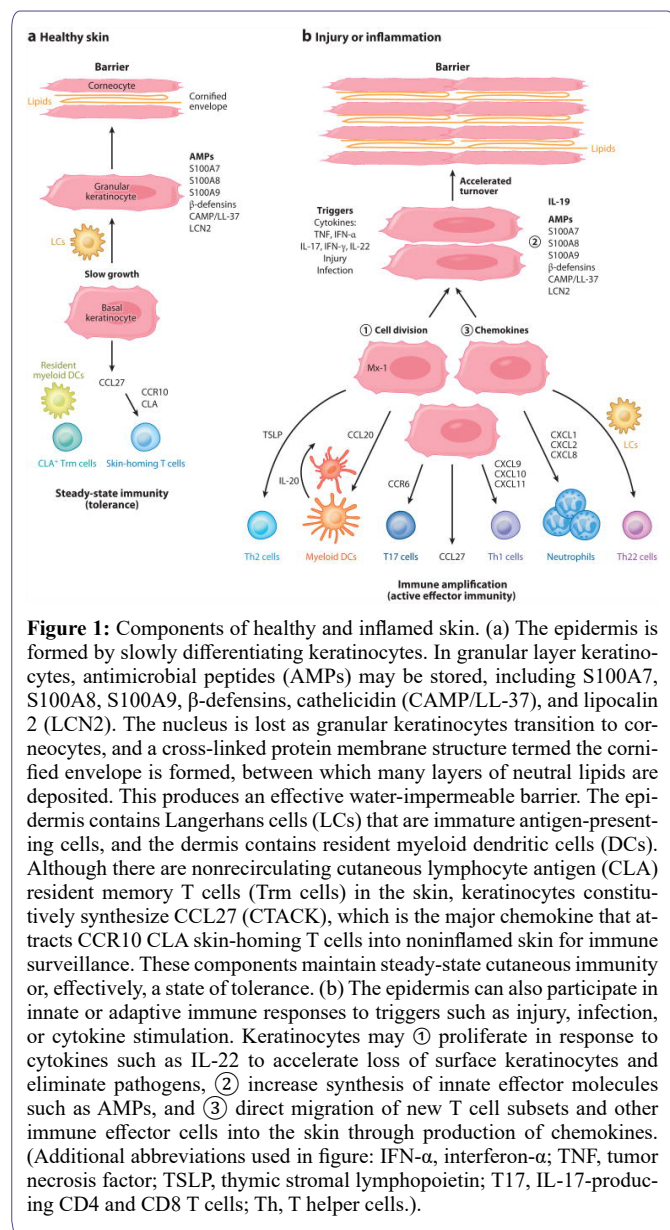
Figure 1: Components of healthy and inflamed skin. (a) The epidermis is formed by slowly differentiating keratinocytes. In granular layer keratinocytes, antimicrobial peptides (AMPs) may be stored, including S100A7, S100A8, S100A9, β-defensins, cathelicidin (CAMP/LL-37), and lipocalin 2 (LCN2). The nucleus is lost as granular keratinocytes transition to corneocytes, and a cross-linked protein membrane structure termed the cornified envelope is formed, between which many layers of neutral lipids are deposited. This produces an effective water-impermeable barrier. The epidermis contains Langerhans cells (LCs) that are immature antigen-presenting cells, and the dermis contains resident myeloid dendritic cells (DCs). Although there are nonrecirculating cutaneous lymphocyte antigen (CLA) resident memory T cells (Trm cells) in the skin, keratinocytes constitutively synthesize CCL27 (CTACK), which is the major chemokine that attracts CCR10 CLA skin-homing T cells into noninflamed skin for immune surveillance. These components maintain steady-state cutaneous immunity or, effectively, a state of tolerance. (b) The epidermis can also participate in innate or adaptive immune responses to triggers such as injury, infection, or cytokine stimulation. Keratinocytes may ① proliferate in response to cytokines such as IL-22 to accelerate loss of surface keratinocytes and eliminate pathogens, ② increase synthesis of innate effector molecules such as AMPs, and ③ direct migration of new T cell subsets and other immune effector cells into the skin through production of chemokines. (Additional abbreviations used in figure: IFN-α, interferon-α; TNF, tumor necrosis factor; TSLP, thymic stromal lymphopoietin; T17, IL-17-producing CD4 and CD8 T cells; Th, T helper cells.).

Therefore, to assess the equilibrium dissociation constant (K_d), the reaction was carried out for 30 min. The nonspecific binding of [³H]Np5 under these conditions was 12.6 ± 0.3 % of total binding. An analysis of the specific binding of [³H]Np5 to the cells in the Scatchard coordinates (Figure 2) showed that there is one class of binding sites (receptors) for this peptide on their surface: the plot represents a straight line. The K_d value equal to 2.6 ± 0.2 nM indicates a high affinity of labeled peptide to the receptor.

To characterize the specificity of the [³H]Np5 binding to the cells, unlabeled TM-α_1, IFN-α_2, Np5, and the peptide iNp5 with the reverse KKEKL sequence were tested as potential competitors. The K_i values (Table 2) demonstrated strong inhibitory capacity of TM-α_1, IFN-α_2, and Np5, whereas iNp5 did not inhibit the labeled peptide binding ($K_i > 10$ μM), indicating a high specificity of TM-α_1, IFN-α_2, and iNp5 binding. Thus, TM-α_1, IFN-α_2, and Np5 bind with high affinity and specificity to the common receptor on normal human keratinocytes.
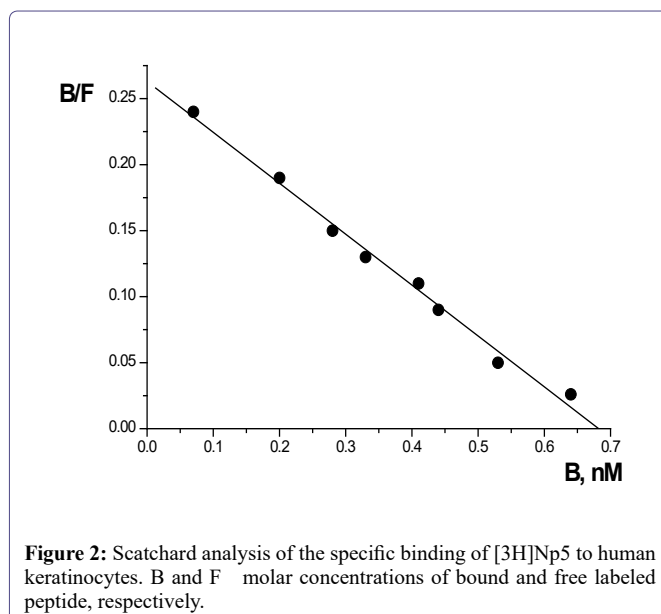

Figure 2: Scatchard analysis of the specific binding of [3H]Np5 to human keratinocytes. B and F molar concentrations of bound and free labeled peptide, respectively.

Ligand	IC50*, nM	Ki*, nM
IFN-α_2	7.2 ± 0.4	2.5 ± 0.2
TM-α_1	7.6 ± 0.4	2.6 ± 0.3
Np5	8.3 ± 0.3	2.9 ± 0.2
iNp5	>100	>100

Table 2: Inhibition of [3H]Np5 specific binding to human keratinocytes by unlabeled ligands.

*Values are means ± SEM of two independent experiments, each performed in triplicates.

Effects of Np5 on sGC and pGC activity of human keratinocytes

The results presented in Table 3 show that Np5 at concentrations of 501000 nM increased in a dose-dependent manner the sGC activity in the cells, but did not affect the pGC activity; iNp5 tested in parallel was inactive. Thus, the activating action of Np5 on sGC was specific and dose-dependent (Table 3).

Effect of Np5 on IL-17A-induced cytokine secretion by human keratinocytes

The results presented in Table 4 show that that pretreatment of cells with Np5 (the concentration range of 50-1000 nM) significantly reduced production of TNF-α, IL-6, and IL-1α, when compared to cells treated with IL-17A alone. Peptide with inverted amino acid sequence iNp5 was inactive; which indicates a high specificity of the Np5 action. Thus, Np5 reduces the production of pro-inflammatory cytokines *in vitro*. The data presented in Table 5 show a significant increase in the secretion of the anti-inflammatory cytokine IL-10 in response to pretreatment of cells by Np5. Tested in parallel iNp5 was inactive. Thus, Np5 increases the secretion of the anti-inflammatory cytokine IL-10 *in vitro*.

Effect of ODQ on inhibitory action of Np5 on IL-17A-induced cytokine secretion by human keratinocytes

The data presented in the Table 6 show that ODQ, an inhibitor of soluble guanylate cyclase [19], suppressed in a dose-dependent

Ligand (nM)	Guanylate cyclase activity (nmoles of cGMP per 1 mg protein in 10 min ± SEM)			
	sGC		pGC	
	Np5	iNp5	Np5	iNp5
Control	1.6 ± 0.2		2.2 ± 0.2	
10	1.7 ± 0.3	1.6 ± 0.2	2.3 ± 0.3	2.0 ± 0.3
50	2.1 ± 0.2*	1.7 ± 0.2	2.4 ± 0.3	2.2 ± 0.3
100	2.4 ± 0.2*	1.6 ± 0.3	2.1 ± 0.2	2.0 ± 0.4
500	2.9 ± 0.3*	1.5 ± 0.3	2.2 ± 0.3	2.3 ± 0.2
1000	2.8 ± 0.3*	1.8 ± 0.3	2.4 ± 0.2	2.0 ± 0.4

Table 3: Effects of Np5 and iNp5 on the sGC and pGC activity of human keratinocytes.

*Significant differences between experience and control ($P<0.05$).

Peptide (nM)	Cytokine (pg/mg protein ± SEM)					
	TNF-α		IL-6		IL-1α	
	Np5	iNp5	Np5	iNp5	Np5	iNp5
Control	28.3 ± 2.3		15.5 ± 1.7		27.4 ± 2.2	
10	29.6 ± 2.5	28.8 ± 2.7	15.9 ± 1.2	15.5 ± 1.6	28.0 ± 2.9	27.1 ± 2.5
50	23.4 ± 2.8*	27.5 ± 3.3	11.6 ± 1.4*	15.9 ± 1.4	24.7 ± 2.6	27.5 ± 2.7
100	19.6 ± 2.5*	28.2 ± 2.9	9.7 ± 1.3*	15.2 ± 1.5	20.7 ± 2.5*	27.7 ± 2.9
500	15.3 ± 2.4*	27.8 ± 2.5	8.5 ± 1.4*	16.0 ± 1.8	17.5 ± 2.4*	27.4 ± 2.8
1000	15.9 ± 2.7*	29.2 ± 2.6	8.8 ± 1.6*	15.7 ± 1.6	18.1 ± 2.9*	27.2 ± 2.3

Table 4: Effect of pretreatment of human keratinocytes with Np5 and iNp5 on IL-17A-induced secretion of pro-inflammatory cytokines.

*Significant differences between experience and control ($P<0.05$).

Peptide (nM)	IL-10 (pg/mg protein ± SEM)	
	Np5	iNp5
Control	33.4 ± 3.2	
10	30.9 ± 3.6	34.0 ± 3.7
50	26.2 ± 3.4*	33.2 ± 3.5
100	23.3 ± 3.3*	32.9 ± 3.3
500	20.5 ± 3.7*	33.7 ± 3.2
1000	21.1 ± 3.0*	33.5 ± 3.4

Table 5: Effect of pretreatment of human keratinocytes with Np5 and iNp5 on IL-17A-induced secretion of anti-inflammatory cytokine IL-10.

*Significant differences between experience and control ($P<0.05$).

ODQ (μM)	Guanylate cyclase activity (nmoles of cGMP per 1 mg protein in 10 min ± SEM)	Cytokine (pg/mg protein ± SEM)	
		TNF-α	IL-1α
Control	2.8 ± 0.2	15.9 ± 2.7	18.0 ± 2.4
5	2.2 ± 0.3*	18.2 ± 2.5*	20.2 ± 2.6
10	1.3 ± 0.3*	20.3 ± 2.2*	24.2 ± 2.3*
50	<0.1*	29.2 ± 2.7*	26.6 ± 2.9*
100	<0.1*	28.5 ± 2.4*	27.0 ± 2.5*

Table 6: Effect of ODQ on the sGC activity and IL-17A-induced cytokine secretion by human keratinocytes pretreatment with 500 nM Np5.

*Significant differences between experience and control ($P<0.05$).

manner the sGC activity as well as the inhibitory effect of 500 nM Np5 on IL-17A-induced TNF-α and IL-1α secretion by human keratinocytes. Thus, inhibition of the sGC activity leads to a loss of the Np5 ability to inhibit the pro-inflammatory cytokine production by the cells of *in vitro*.

Discussion

Screening for the activity of 36 cytokines identified five pro-inflammatory cytokines IL-17A, IL-22, IL-20, IL-6, IL-8, on costatin M (OSM), TNF-α, IL-1α, IL-1β as potent inducers of skin inflammation, of which IL-1a, IL-17A, and TNF-a showed the highest activity [1]. A year ago, 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* [8]. In this study we examined the anti-inflammatory potential of Np5 using an *in vitro* model of IL-17A-induced inflammation in normal human keratinocytes. For this, cells were treated with the peptide in the concentration range of 101000 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 501000 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) (Table 4), and at the same time, enhanced the production of anti-inflammatory Il-10 (Table 5). The peptide with inverted amino acid sequence iNp5 was inactive; which indicates a high specificity of the Np5 action.

We recently 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 [4,8,9]. The results of the present study show that Np5 binds with high affinity to human keratinocytes (K_d 2.6 nM) and increases in a dose-dependent manner the sGC activity (Table 3). 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 [19]. 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 (Table 6). Therefore, Np5 reduces the pro-inflammatory cytokine secretion in IL-17A-stimulated human keratinocytes via sGC.

Conclusion

Np5 (LKEKK) binds with high specificity and affinity to human keratinocytes, reduces their ability to the IL-17A-induced secretion of proinflammatory cytokines (TNF-α, IL-6, and IL-1α) and increases their production of anti-inflammatory cytokine IL-10. It should be noted that Np5, which is a short peptide with a simple structure, has significant potential as an anti-inflammatory drug.

Declaration of Interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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