ВЛИЯНИЕ ЭНДОМОРФИНОВ НА ГУМОРАЛЬНЫЙ ИММУННЫЙ ОТВЕТ, ПРОДУКЦИЮ Th1/Th2/Th17-ЦИТОКИНОВ И АПОПТОЗ CD4$^+$, CD8$^+$ ЛИМФОЦИТОВ *IN VIVO*

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Резюме. Эндогенные опиоидные пептиды представляют собой большую группу физиологически активных соединений с выраженным сродством к рецепторам опиоидного типа, способную проявлять выраженную анальгетическую активность, а также оказывать дополнительные эффекты на периферии, ввиду своего широкого распространения на клетках многих органов и тканей. Мало изученными представителями этой группы являются эндоморфины, которые, благодаря своей структуре и свойствам, способны производить сильное антиноцицептивное воздействие после центрального введения, а значит, в перспективе, они могут рассматриваться как потенциальные заменители низкомолекулярных опиатов. Цель данного исследования заключается в том, чтобы оценить влияние эндоморфинов на гуморальный иммунный ответ, продукцию Th1/Th2/Th17-цитокинов и апоптоз CD4$^+$, CD8$^+$ лимфоцитов *in vivo*. В качестве объекта исследования использовали спленоциты белых мышей самцов породы Swiss. Оценку количества антителообразующих клеток в селезенке проводили с использованием метода локального гемолиза в геле агарозы по Jerne. Количественное определение цитокинов проводили методом твердофазного иммуноферментного анализа с помощью наборов (R&D, США) согласно методике, предложенной производителем. Апоптоз оценивали при помощи реагентов Annexin V-FITC/7-AAD kit (Beckman Coulter, США) в соответствии с инструкцией производителя методом проточной цитометрии на проточном цитофлюориметре CytoFLEX S (BeckmanCoulter, США). В ходе исследования установлено, что эндоморфины усиливают антителогенез селезенки, а предварительная блокада опиатных рецепторов налоксоном приводила к отмене стимулирующего влияния пептидов. Эндоморфины не влияли на продукцию спленоцитами IL-2, IL-4, IFNγ, однако введение эндоморфина-2 налоксоннезависимо усиливало индуцированную продукцию IL-17. Оценка влияния эндоморфинов на апоптоз спленоцитов 24 ч культур показала, что эндоморфин-2 в нестимулированных культурах налоксонзависимо увеличивал процент позднего апоптоза CD8$^+$ лимфо-

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цитов, однако в индуцированных культурах оба эндоморфина повышали апоптотическую активность CD8+ лимфоцитов уже независимо от предварительной блокады опиоидных рецепторов. Подводя итог, можно сказать, что в системе *in vivo* эндоморфины оказывают широкий спектр разнонаправленных иммуномодулирующих эффектов, которые в дальнейшем могут представлять большой интерес для практического использования.

Ключевые слова: эндоморфины, спленоциты, антителообразующие клетки, апоптоз, цитокины, налоксон

EFFECT OF ENDOMORPHINS ON HUMORAL IMMUNE RESPONSE, Th1/Th2/Th17 CYTOKINE PRODUCTION AND CD4+, CD8+ LYMPHOCYTE APOPTOSIS *IN VIVO*

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Abstract. Endogenous opioid peptides are a large group of physiologically active compounds with a pronounced affinity for opioid-type receptors, capable of showing pronounced analgesic activity, as well as having additional effects on the periphery, due to their wide distribution on the cells of many organs and tissues. Little studied representatives of this group are endomorphins, which due to their structure and properties, are capable of producing a strong antinociceptive effect after central administration, which means that, in the future, they can be considered as potential substitutes for low molecular weight opiates. The aim of this study is to evaluate the effect of endomorphins on the humoral immune response, the production of Th1/Th2/Th17 cytokines and apoptosis of CD4+, CD8+ lymphocytes *in vivo*. The splenocytes of Swiss white mice were used as the object of the study. The number of antibody-forming cells in the spleen was assessed using the method of local hemolysis in agarose gel according to Jerne. Quantitative determination of cytokines was carried out by enzyme-linked immunosorbent assay using kits (R&D, USA) according to the method proposed by the manufacturer. Apoptosis was assessed using Annexin V-FITC/7-AAD kit reagents (Beckman Coulter, USA) according to the manufacturer's instructions by flow cytometry on a CytoFLEX S flow cytometer (Beckman Coulter, USA). In the course of the study, it was found that endomorphins enhance the antibody genesis of the spleen, and the preliminary blockade of opiate receptors with naloxone led to the cancellation of the stimulating effect of peptides. Endomorphins didn't affect splenocyte production of IL-2, IL-4, and IFNγ, however, the introduction of endomorphin-2 naloxone-independent enhanced the induced production of IL-17. Evaluation of the effect of endomorphins on apoptosis of splenocytes in 24-h cultures showed that endomorphin-2 in unstimulated cultures of naloxone-dependently increased the percentage of late apoptosis of CD8+ lymphocytes, however, in stimulated cultures, both endomorphins increased the apoptotic activity of CD8+ lymphocytes, regardless of the preliminary blockade of opioid receptors. In summary, we can say that in the *in vivo* system, endomorphins have a wide range of multidirectional immunomodulatory effects, which may be of interest for practical use in the future.

Keywords: endomorphins, splenocytes, antibody-forming cells, apoptosis, cytokines, naloxone

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Introduction

Endogenous opioid peptides are a large group of physiologically active compounds with a pronounced affinity for opioid-type receptors, capable of showing pronounced analgesic activity, as well as having additional effects on the periphery, due to the wide expression of opioid receptors on the cells of many organs and tissues [3, 4, 13]. It is well known that μ-opioid receptor agonists are the most effective means of relieving severe pain. Their antinociceptive action is due to activity at the supraspinal, spinal and peripheral levels [6]. Endomorphins are tetrapeptides that are highly affine selective agonists of the μ-receptor. Based on their structure, these peptides

represent a potential "substitute" for low-molecular-weight opiates [9, 12]. They act like morphine, but presumably have fewer side effects caused by low-molecular-weight opiates.

From a practical point of view, the introduction of endogenous antinociceptive ligands can be accompanied by a number of advantages, such as rapid enzymatic degradation and low toxicity [6, 7, 10, 11]. Therefore, in the literature, selective μ-receptor ligands are considered as promising compounds, and their wide distribution in the immune system suggests an active participation in the modulation of the functions of innate and adaptive immunity cells [6, 9, 15].

The aim of the study was to evaluate the effect of endomorphins on the humoral immune response, the production of Th1/Th2/Th17 cytokines and apoptosis of $CD4^+$, $CD8^+$ lymphocytes *in vivo*.

Materials and methods

Experimental studies were carried out on white Swiss male mice with a body weight of 20±2 g. All experiments were conducted strictly in accordance with the recommendations and ethical standards specified in the "European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes" (Strasbourg, 1986), and also comply with the ethical standards approved by the legal acts of the Russian Federation.

Endomorphins (Sigma, USA) were administered intraperitoneally to mice at a dose of 100 μg/kg, control animals were injected with 0.9% NaCl in the appropriate volume. Naloxone hydrochloride (Moscow Endocrine Factory, Russia) was injected subcutaneously at a dose of 0.2 μg/kg 20 min before the administration of endomorphins.

To assess the effect of endomorphins on the formation of antibody-forming cells (AFC), the spleens of animals were immunized with ram erythrocytes (ER, NPO Microgen, Russia) intraperitoneally (10^8 cells in 0.2 mL of 0.9% NaCl) after 60 minutes from the moment of administration of endomorphins. On the 5th day, the animals were taken out of the experiment by decapitation under ether anesthesia, and the amount of AFC was determined by local hemolysis in an agarose gel according to Jerne [8].

To study the effects of endomorphins on the production of IL-2, IL-4, IL-17, and IFNγ, mice were removed from the experiment 1 hour after the introduction of peptides and their spleen was extracted, followed by the isolation of splenocytes. Cells were cultured in 96-well plates (1×10^7cells/1 mL of complete nutrient medium, which was prepared on the basis of RPMI 1640 medium (Gibco, UK) with the addition of 10 mM HEPES (Sigma, USA), 2mM Glutamax (Sigma-Aldrich, USA), 100 units/mL gentamicin and 10% embryonic calf serum (Capricorn scientific, Germany)) at 37 °C for 24 hours. ConA (MPBiomedicals, France) at a concentration of 10 μg/mL was used as an inducer. Culture supernatants were collected, frozen and stored at -20 °C. The determination of IL-2, IL-4, IL-17, IFNγ was carried out by enzyme-linked immunosorbent assay using R&D kits (USA) according to the method proposed by the manufacturer.

The study of the effect of endomorphins on the apoptotic activity of $CD4^+$, $CD8^+$ lymphocytes was performed on the day of the experiment and in 24 h cultures. Animals were removed from the experiment 1 hour after the administration of the peptide; the isolated splenocytes were stained with monoclonal antibodies PE anti-mouse $CD4^+$ and PE anti-mouse $CD8^+$ (BioLegend, USA) for 20 minutes in accordance with the instructions provided by the manufacturer. Apoptosis was assessed using Annexin V-FITC/7-AAD kit reagents (Beckman Coulter, USA). The results were recorded by flow cytometry on a CytoFLEX S flow cytometer (Beckman Coulter, USA). To assess the effect of endomorphins on apoptosis of cell cultures, isolated splenocytes were cultured in 96-well plates (4×10^6 cells/mL of complete nutrient medium, which was prepared on the basis of RPMI 1640 medium (Gibco, UK) with the addition of 10 mM HEPES (Sigma, USA), 2 mM Glutamax (Sigma-Aldrich, USA), 100 units/mL gentamicin and 20% embryonic calf serum (Capricorn scientific, Germany), 10 μM 2-mercaptoethanol (Gibco, USA)) at 37 °C for 24 h in the presence of ConA 10 μg/mL.

Statistical data processing was carried out using the Student's unpaired t-test. The differences were considered significant at $p < 0.05$. The results are presented in the form of the arithmetic mean and its standard error (M±m).

Results and discussion

Evaluation of the effect of endomorphins on the amount of AFC showed that intraperitoneal administration of peptides significantly increased both the absolute and relative number of antibody-forming cells compared with the control (Table 1). Preliminary blockade of opiate receptors with naloxone led to the abolition of the stimulating effect of endomorphins.

Analysis of the effect of endomorphins on cytokine production showed that neither the peptides themselves nor their administration against the background of opioid receptor blockade had any effect on spontaneous and stimulated production of IL-2, IL-4, IFNγ (data not provided). At the same time, endomorphins increased the stimulated production of IL-17 (Figure 1); this effect was not canceled by the administration of naloxone.

In experiments to study the effects of endomorphins on $CD4^+$, $CD8^+$ lymphocyte apoptosis, no statistically significant effects were detected imme-

TABLE 1. EFECT OF ENDOMORPHIN-1, ENDOMORPHIN-2, AND NALOXONE ON THE ABSOLUTE AND RELATIVE NUMBER OF AFC IN THE SPLEEN

Effect	Log_{10} AFC/million	Log_{10} AFC/organ
Control (n = 10)	1.46±0.20	3.87±0.22
Endomorphin-1 (n = 6)	1.96±0.08*	4.63±0.14*
Endomorphin-2 (n = 8)	2.02±0.11*	4.47±0.16*
Endomorphin-1 + naloxone (n = 6)	1.36±0.09	3.78±0.07
Endomorphin-2 + naloxone (n = 6)	1.81±0.13	4.17±0.13
Naloxone (n = 8)	1.91±0.08	4.23±0.17

Note. *, $p < 0.05$ compared to the control. AFC, Antibody-forming cells.

diately after the animals were removed from the experiment (data not provided). Evaluation of the effect of endomorphins on apoptosis of splenocytes in 24-hour cultures showed that endomorphin-2 in unstimulated cultures increased the percentage of late apoptosis of $CD8^+$ lymphocytes, and the preliminary administration of naloxone led to the abolition of this effect (Table 2). In stimulated cultures, both endomorphins also significantly increased the apoptotic activity of $CD8^+$ lymphocytes, however, in this case, the effect of endomorphins on the background of naloxone administration did not change.

Thus, endomorphins *in vivo* naloxone-dependent stimulate antibody formation and enhance $CD8^+$ lymphocyte apoptosis and IL-17 production independently of opioid receptor blockade. Previously, we have shown that *in vivo* administration of β-endorphin to mice at doses of 1; 0.01 and 0.0005 μg/kg statistically significantly increased the amount of AFC in the spleen, however, this effect was not reversed by naloxone [5]. In the *in vitro* system, the addition of endomorphins to splenocytes of mice led to naloxone-independent inhibition of the production of antibodies against sheep erythrocytes, at the same time the effect was leveled by the introduction of monoclonal antibodies to endomorphins [1].

Despite the modulation of the humoral immune response by endomorphins, the effects of peptides on the synthesis of Th1/Th2 cytokines (IL-2, IL-4, IFNγ) could not be identified, however, an effect on the synthesis of IL-17, a cytokine that plays a key role in protecting organism from extracellular bacterial and fungal infections. Previously, data on the participation of endomorphins in the regulation of IL-17 production were not mentioned in the literature. In addition, endomorphins naloxone-independently enhanced late apoptosis of $CD8^+$ lymphocytes. It can be assumed that the implementation of inhibitory signals in relation to the effector link of cellular immunity can be mediated by stimulation of the μ-receptor. It is known that $CD8^+$T lymphocytes are more sensitive to the apoptosis-inducing action of the peptides studied by us, compared with $CD4^+$ cells [2].

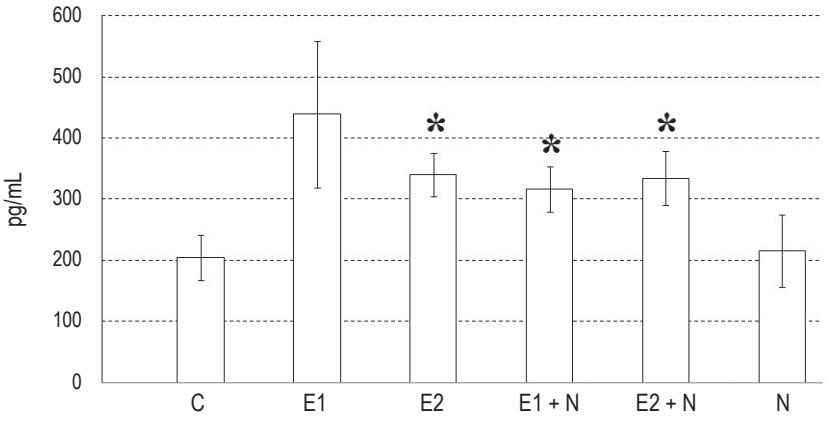

Figure 1. Effect of endomorphins-1, 2 and naloxone on IL-17 production in stimulated splenocytes
Note. *, $p < 0.05$ compared to the control (n = 9). C, Control; E1, Endomorphin-1; E2, Endomorphin-2; N, Naloxone; E1 + N, Endomorphin-1 + naloxone; E2 + N, Endomorphin-2 + naloxone.

TABLE 2. EFFECT OF ENDOMORPHINS-1, 2 AND NALOXONE ON EARLY AND LATE APOPTOSIS OF $CD4^+$ AND $CD8^+$T LYMPHOCYTES AFTER 24 h CULTIVATION

Effect	CD4		CD8	
	Early	Late	Early	Late
Without inductor				
Control	6.93±0.45	18.86±3.01	3.63±0.33	19.36±1.77
Endomorphin-1	8.73±1.44	20.38±2.11	3.68±0.48	21.76±3.35
Endomorphin-2	7.21±0.82	20.06±1.22	4.28±0.45	25.49±1.95*
Endomorphin-1 + naloxone	8.67±1.07	21.01±2.79	3.93±0.24	27.98±3.39*
Endomorphin-2 + naloxone	6.85±1.35	25.22±2.27	4.18±0.53	23.03±2.22
Naloxone	7.75±0.48	23.34±2.38	4.66±0.46	23.71±2.75
ConA				
Control	8.44±1.19	35.61±11.2	4.76±0.45	18.17±1.34
Endomorphin-1	7.41±0.46	29.82±5.15	4.74±1.29	34.26±6.36*
Endomorphin-2	8.48±0.53	19.63±1.99	3.83±0.47	28.58±2.82*
Endomorphin-1 + naloxone	7.78±0.49	22.39±1.91	4.65±0.44	32.59±4.55*
Endomorphin-2 + naloxone	5.43±1.15	31.28±6.11	6.30±0.69	44.34±4.13*
Naloxone	8.34±0.83	17.76±3.58	4.75±0.57	21.45±0.99

Note. *, $p < 0.05$ compared to the control (n = 6); ConA, concanavalin A.

Naloxone, unlike endomorphins, is a complete antagonist of the μ-receptors, displacing mainly morphine derivatives from the binding site within the receptor, thereby eliminating their effect on the body. However, the blockade of opioid receptor may not always lead to the abolition of the action of agonists, especially of a peptide nature. The reversal of the effect of agonists by naloxone may depend on a number of factors, such as ligand concentration, experimental model, age and gender. Thus, it was shown that cold stress-induced analgesia was naloxone-dependent in females and naloxone-independent in males [14].

Conclusion

In summary, we can say that in the *in vivo* system, the effects of endomorphins are comparable in nature to the immunomodulatory effects of other opioid peptides, in particular endorphins, and have little in common with the effects of morphine, which has a pronounced inhibitory effect on the functions of innate and adaptive immunity cells. This proves that there are significant differences in the mechanism of immunomodulatory action between opioid agonists of a peptide and non-peptide nature.

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