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Anti-Inflammatory Activity of a Polypeptide from the *Heteractis crispa* Sea Anemone[1]

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Abstract—The anti-inflammatory activity of the HCGS 1.20 recombinant polypeptide (a Kunitz-type serine protease inhibitor from the *Heteractis crispa* sea anemone) was investigated. The polypeptide was shown to inhibit the histamine-induced increase in the concentration of calcium ions and the lipopolysaccharide-stimulated increase in the concentration of nitric oxide (II) in macrophages. A possible mechanism of this anti-inflammatory activity of the polypeptide was discussed.

Keywords: sea anemones, the Kunitz-type protease inhibitors, antihistamine activity, anti-inflammatory activity

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INTRODUCTION

The inflammatory process is a basis of many diseases. An inflammation is almost always developed in an organism in response to the penetration of molecules foreign for the host immune system. A septic (microbial) inflammation appears in the case of affection of an organism with pathogenic bacteria. This inflammation is mainly mediated by the organism intoxication with bacterial lipopolysaccharides (LPSs). LPSs interact with specific Toll-like receptors on a surface of immunocompetent cells, initiate a fast response, and, depending on the receptor type and the way of signal transduction, induce synthesis of inflammatory cytokines or activate expression of the genes that determine the cellular inflammatory response. A search for compounds which can inhibit undesired inflammatory-inducing enzymatic activity or the receptors or/and ionic channels that are involved in these processes is a promising approach to the treatment of such pathologies.

At present, a large number of natural protease inhibitors of various types are known. They are found both in invertebrates and mammals. The Kunitz-type protease inhibitors are of special interest because of their wide occurrence in various organisms, structural diversity, and multifunctionality. Sea anemones are one of sources of the Kunitz-type protease inhibitors [1, 2]. The APHC1 and APHC3 polypeptides from the *Heteractis crispa* tropical sea anemone have been found to block the TRPV1 pain vanilloid receptor and to exhibit an analgesic effect in vivo without hyperthermia [3, 4] along with their blocking of the trypsin and chymotrypsin activity. A possible joint effect of these peptide inhibitors on receptors and proteases that participate in the inflammatory processes has been proposed [5, 6]. Previously, an antihistamine activity has been found for the RmIn I and RmIn II from *H. crispa* in the in vivo experiments, suggesting their possible participation in anti-inflammatory processes.

The goal of this study is the investigation of the anti-inflammatory effect of the HCGS 1.20 recombinant polypeptide, which is a Kunitz-type inhibitor of serine proteases and a product of expression of the *hcgs 1.20 H. crispa* gene, on the in vitro models.

RESULTS AND DISCUSSION

The HCGS 1.20 polypeptide is a representative of the combinatory library of the Kunitz-type inhibitors of serine proteases from the *H. crispa* sea anemone. It was produced by expression of the *hcgs 1.20* gene in a bacterial system. The hybrid protein contains the HCGS 1.20 polypeptide and thioredoxin for the correct formation of disulfide bonds. It was isolated from the cellular lysate by an affinity chromatography, treated with cyanogen bromide for the cleavage of the polyhistidine sequence and thioredoxin, and purified by reversed phase HPLC. The yield of the recombinant polypeptide proved to be 8.2 mg/L of the cellular culture. The HCGS 1.20 molecular mass was 6078.9 Da according to the MALDI TOF mass spectrometry. This value was in a good agreement with the calculated

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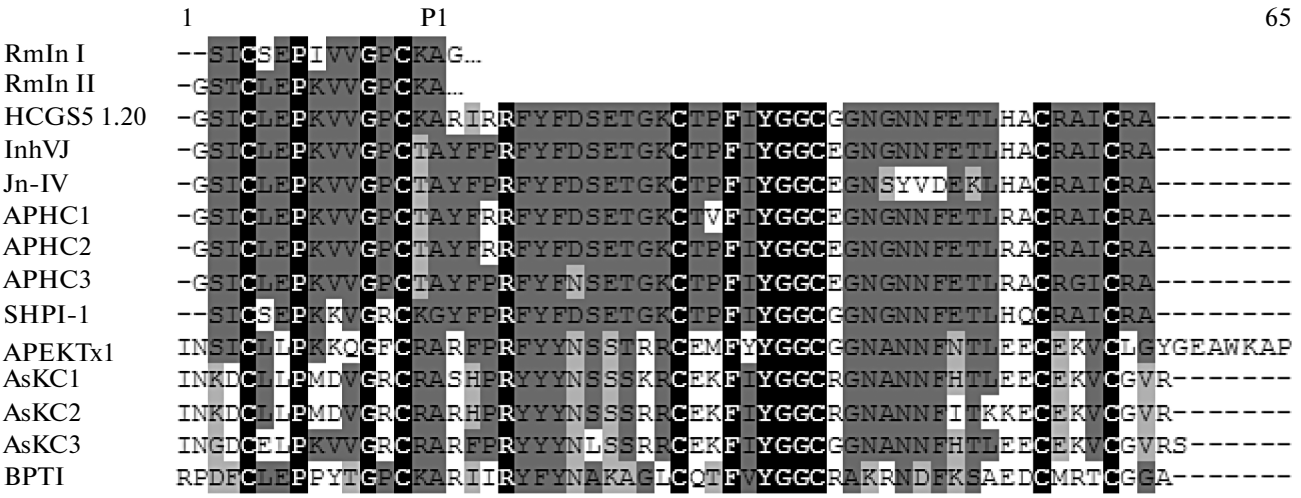

Fig. 1. Amino acid sequences of the inhibitors from sea anemones and bovine pancreatic inhibitor of trypsin. RmIn I, RmIn II [7], InhVJ [8], Jn-IV [9], and APHC1–APHC3 [3, 4] from *H. crispa*; SHPI-1 and SHPI-2 from *Stichodactyla helianthus* [10]; AsKC1–AsKC3 from *Anemonia sulcata* [11]; APEKTx1 from *Anthopleura elegantissima* [12]; BPTI from *Bos taurus* [13]. *P*1 is an amino acid residue of the active site of the inhibitors. Conservative and highly homologous amino acid residues are colored by dark and light gray, respectively. The identical residues are printed against the black background.

molecular mass (6080.0 Da). The *N*-terminal amino acid sequence of HCGS 1.20 (20 amino acid residues) was determined by the Edman automatic sequencing and appeared to be identical to that evaluated on the basis the gene sequence (Fig. 1).

Previously, we have shown that the serine protease inhibitors from the *H. crispa* sea anemone form a combinatory library of the Kunitz-type polypeptides. Representatives of this library are encoded by different multigene families [14]. According to the phylogenetic analysis, the inhibitors are divided into three groups which are distinguished by amino acid residues in position *P*1 of the protease binding site. The polypeptides of the largest group I (66.1%) contain the positively charged lysine residue similarly to the bovine pancreatic trypsin inhibitor (BPTI) [13]. Representatives of group II and III have the threonine (the analgesic cluster) and arginine residues, respectively. The APCH1–APCH3, HCGS 1.10, and HCGS 1.36 polypeptides are found to exhibit the analgesic activity [3, 4, 15]. Amino acid sequence of polypeptides of group III is highly homologous to that of SHPI-1 inhibitor from the *Stichodactyla helianthus* sea anemone which exhibits wide specificity to proteases, including serine (trypsin, chymotrypsin, and human neutrophil elastase), cysteine (papain), and asparagine (pepsin) proteases [10].

The HCGS 1.20 polypeptide belongs to group I (Lys in position *P*1) according to the phylogenetic analysis, is highly homologous to the most of the known polypeptides of the Kunitz-type from sea anemones (up to 91%) and has maximum degree of the gene expression at the RNA level (18.5%) [14]. The inhibition constants of HCGS 1.20 for trypsin and α-chymotrypsin are determined by the Dixon method and proved to be 2.1×10^{-8} and 5.0×10^{-7} M, respectively. These values are six-order higher than the BPTI constant and comparable with the calculated constants of polypeptides from sea anemones (table).

The HCGS 1.20 polypeptide exhibits the analgesic activity comparable with that of the HCGS 1.36 inhibitor [15]. The RmIn I and RmIn II inhibitors of group I are known to have the antihistamine activity and reduce clinical presentations of allergy, and the observed effect is dose-dependent [7]. In this connection, we examine the antihistamine and anti-inflammatory activity of the HCGS 1.20 polypeptide in this study.

The anti-inflammatory effect of the HCGS 1.20 polypeptide was evaluated according to its influence on the LPS-induced production of nitric oxide (II) in the mouse macrophages of the RAW 264.7 cellular line by the method [17]. The 24-h incubation of the cells in the presence of LPS resulted in the significant increase in the NO level, whereas the addition of the HCGS 1.20 peptide in a concentration of 10 μM decreased the NO content by 25% (Fig. 2). Synthesis of nitric oxide (II) is known to occur after the activation of the inducible NO-synthase (iNOS) in a cytoplasm [18–20]. NO participates in regulation of the NADPH-oxidase system and plays an important role in the system of cellular immunity and modulation of the immune response [21]. The NO-excess results in serious diseases at an over-stimulation of the system and redundant inflammatory response [22, 23].

Nowadays, a number of synthetic and natural compounds which are able to considerably decrease the NO content in the RAW 264.7 macrophages have been

Inhibition constants of trypsin and α-chymotrypsin by the protease inhibitors from sea anemones which were calculated by the Dixon method

Source	Polypeptide	K_i of trypsin, M	K_i of chymotrypsin, M	Literature
Heteractis crispa	HCGS 1.20	2.1×10^{-8}	5.0×10^{-7}	
	RmIn I	2.4×10^{-9}	2.3×10^{-8}	[7]
	RmIn II	2.5×10^{-9}	3.0×10^{-8}	[7]
	InhVJ	2.5×10^{-9}	9.93×10^{-7}	[8]
	Jn-IV	9.6×10^{-9}	n.d.	[9]
	APHC1	1.0×10^{-6}	5.0×10^{-6}	[3]
	APHC2	9.0×10^{-7}	4.5×10^{-6}	[4]
	APHC3	5.0×10^{-7}	7.0×10^{-6}	[4]
Anemonia sulcata	AsKC1–3	$<3.0 \times 10^{-10}$	n.d.	[11]
Anthopleura elegantissima	APEKTx1	1.2×10^{-7}	n.d.	[12]
Stichodactyla helianthus	SHPI-1	1.1×10^{-10}	2.3×10^{-9}	[10]
Bos taurus	BPTI	6.0×10^{-14}	1.8×10^{-13}	[13, 16]

n.d. means not determined.

found. Derivatives of benzocycloheptoxazine inhibit the NO production in the cells due to the blocking of their LPS-induced activation [25], whereas omega-3-fatty acids decrease the expression level of iNOS [24].

One of the first processes during induction of the inflammation, including the LPS-induced inflammation, is a histamine release from granules of the mast cells. Further, histamine interacts with the histamine receptors. The action of this mediator is revealed even several seconds after the damage and results in the fast development of a pathological process. Therefore, a search for compounds that selectively block the histamine receptors of different types and prevents the histamine binding to its receptors is an urgent problem.

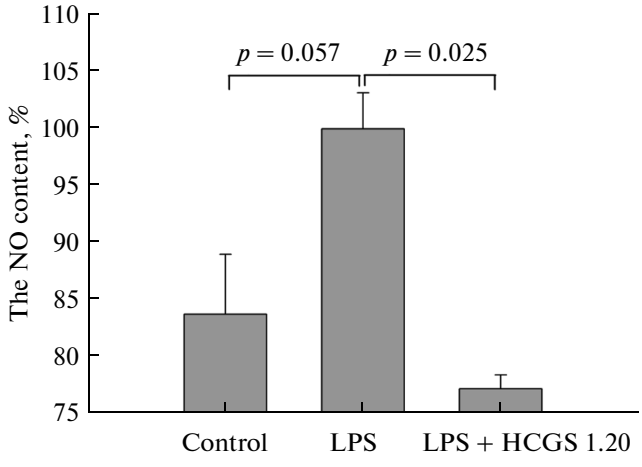

Fig. 2. Effect of the HCGS 1.20 polypeptide on the content of the intracellular NO in the culture of the LPS-stimulated RAW 264.7 mouse macrophages. The data are presented as mean ± standard deviation ($n = 4$).

Macrophages mainly contain the G-protein-coupled histamine receptors of the H_1-type. The histamine interaction with this receptors causes a release of Ca^{2+} ions from the intracellular depots owing to the appearance of inositol-1,4,5-triphosphate in the cytoplasm [26].

The interaction of the HCGS 1.20 recombinant polypeptide with the histamine receptors was investigated on mouse bone marrow-derived macrophages, because these cells had the histamine receptors sensitive to the standard blockers of the H_1-type receptors [27, 28]. The inhibiting effect of the blockers of histamine receptors can be evaluated from the change in the Ca^{2+} concentration ($[Ca^{2+}]_i$) in the cytoplasm of the cells. The macrophages were loaded with the Fluo-3/AM calcium-sensitive fluorescent probe and the change in the intensity of its fluorescence was measured before and after the histamine introduction into the incubation medium for the determination of the Ca^{2+} concentration in the cytoplasm.

Histamine in a concentration of 50 μM caused a significant $[Ca^{2+}]_i$ increase in the cellular cytoplasm within 5–10 s with the subsequent exposure to plateau or slow decay (Fig. 3). Such dynamics of the $[Ca^{2+}]_i$

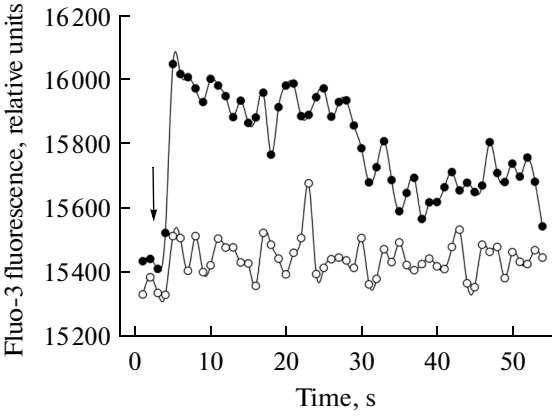

Fig. 3. The effect of histamine in a concentration of 50 μM on the dynamics of the change in fluorescence of the mouse bone marrow-derived macrophages with the Fluo-3/AM calcium-sensitive probe in the control medium (black color) and in the presence of fexofenadine (10 μM) (white color). The time of the mediator introduction is indicated by the arrow.

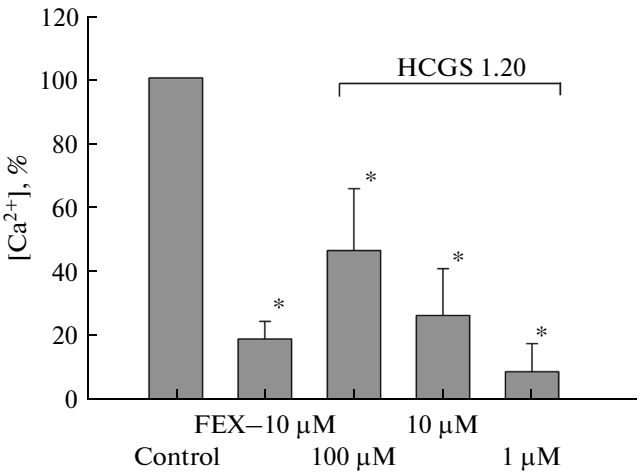

Fig. 4. Effect of histamine (50 μM), fexofenadine (FEX, 10 μM), and the HCGS 1.20 polypeptide in concentrations 1–100 μM on the content of the intracellular Ca^{2+} in the mouse bone marrow-derived macrophages. The data are presented as mean ± standard deviation ($n = 4$). *$p < 0.05$.

change was in a good agreement with the literature data on the histamine influence on functions of the macrophages of human lungs and change in the intracellular concentration of Ca^{2+} under histamine action [27].

Fexofenadine that is a known blocker of the H_1 histamine receptors was used as a positive control. It caused the 80% inhibition of the Ca^{2+}-response to histamine in a concentration of 10 μM (Fig. 4). Incubation of the cells with HCGS 1.20 also resulted in the significant decrease in the Ca^{2+} concentration. The effect manifested itself as a pronounced decrease in the amplitude of the calcium signal in response to the histamine application and, thus, in the lesser change in the intercellular concentration of Ca^{2+}-ions. The inversely proportional dependence of the effect on the HCGS 1.20 concentration was observed; the maximum decrease (90%) was at the polypeptide concentration of 1 μM (Fig. 4).

The ability of low concentrations of HCGS 1.20 to significantly inhibit the histamine effect on the increase in the intracellular Ca^{2+} concentration in the macrophages gives evidence that the polypeptide can block the histamine receptors of the H_1-type and prevent their interaction with histamine.

Thus, these data demonstrate that the HCGS 1.20 polypeptide is a promising anti-inflammatory compound which effectively decreases the effect of such powerful inflammatory mediators as histamine and bacterial LPS in the in vitro experiments.

EXPERIMENTAL

The following reagents were used in this study: the Fluo-3/AM and FA-OMe fluorescent probes (Invitrogen, United States), LPS from *E. coli* of the 055:B5 serotype, histamine, fexofenadine (Sigma, United States), the Miniprep Kit for the plasmid isolation, restriction endonucleases (Fermentas, Lithuania), Ni-NTA-agarose (Qiagen, the Netherlands), components for medium for bacterial culturing (Difco, United States), the XL-1 Blue strains (Stratagene, United States), and BL21 (DE3) *E. coli* (Novagen, Germany). The oligonucleotides were synthesized by ZAO Evrogen (Russia). The solutions were prepared using MilliQ deionized water.

The homologous sequences were searched using the BLAST server [29]. Multiple alignments of the amino acid sequences were carried out using the Vector NTI Advance 11 software (Invitrogen, United States). The sequences of the recombinant plasmids were determined with the application of the standard T7-praimers by the Sanger method on an ABI 313x1 genetic analyzer (Applied Biosystems, United States) according to the manufacturer's program.

Molecular masses of the polypeptides were determined on an Ultraflex III TOF/TOF time-off-flight mass spectrometer (Bruker Daltonic, Germany). The time-off-flight mass spectra were recorded in a direct flight in the reflector regime.

The amino acid sequence of the *N*-terminal fragment of the alkylated recombinant polypeptide was determined on a Procise 492 cLC automatic solid phase amino acid sequencer of proteins (Applied Biosystems, United States) according to the manufacturer's program.

Preparation of the recombinant plasmid for expression of the polypeptide-encoding *hcgs 1.20* gene in *E. coli*. Recombinant DNAs were cloned by the standard methods in the *E. coli* XL-1 Blue cells. The genes were amplified by PCR using the Go-Taq-

polymerase under the conditions that were recommended by the manufacturer of the enzyme. For the preparation of the expression construct, the DNA fragment that encoded the mature HCGS 1.20 was amplified with the gene-specific primers (5'–3': GCGAATTCGATGGGTAGCATTTGTTTAGAACC (Inh_EcoR1_F) and ACTCGAGTTACGCCCTG-CATATAGCTCGGCAT (Inh_XhoI_Rev)) under the following conditions: 3 min at 94°C, 25 cycles: 30 s at 94°C, 30 s at 55°C, 45 s at 72°C, and 15 min at 72°C. The pTZ57R/T recombinant plasmid with the insertion of the *hcgs 1.20* gene was used as a matrix [14]. The PCR product was treated with the EcoRI and XholI endonucleases and cloned into the pET32b(+) vector in the EcoRI and XhoI restriction sites. The pET32b(+)/*hcgs 1.20* plasmids were produced. The recombinant plasmids were separated using the Miniprep Kit according to the manufacturer's instructions and subjected to sequencing. The plasmids with the correct insertion were used for transformation of the *E. coli* cells of the BL21 (DE3) strain by electroporation on a Multiporator (Eppendorf, Germany).

Preparation of the recombinant polypeptide. The transformed cells were cultured in the LB medium (1 L) containing ampicillin (100 μg/mL) at 37°C to the optical density of A_{600} 0.6–0.8. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the final concentration of 0.2 mM for the expression induction. The cells were grown for 16–18 h at 18°C for the production of the recombinant peptide in a soluble form. The bacterial cells were precipitated from the solution by centrifugation at 8000 rpm for 8 min. The conditions for the expression were experimentally found.

The hybrid protein that contained thioredoxin and the HCGS 1.20 polypeptide was isolated by the metal-affinity chromatography on the Ni-NTA-agarose in the native condition from the soluble fraction of the cellular lysate after its ultrasound treatment according to the manufacturer's instruction. Thioredoxin was cleaved by the treatment with cyanogen bromide according to the modified technique [30]. The recombinant HCGS 1.20 peptide was purified by the reversed phase HPLC on a Jupiter C_4 column (10 × 250 mm, Phenomenex, United States) in a concentration gradient of acetonitrile in 0.1% TFA (pH 2.2) from 0 to 70% within 70 min at a flow rate of 3 mL/min. The retention time of the target product was 28 min. The HCGS 1.20 molecular weight was 6078.9 Da.

The disulfdide bonds were reduced by the following procedure. The 1.4 M solution of dithiotreitol (2 μL) was added to the solution of the recombinant polypeptide (100 μg) in 6 M guanidine hydrochloride (70 μL) in 0.5 M Tris-HCl (pH 8.5) with 2 mM EDTA. The reaction mixture was incubated for 4 h at 40°C. The thiol groups of the cysteine residues were modified by the treatment with the 50% solution (2 μL) of 4-vinylpyridine in isopropanol for 20 min at room temperature in the dark. The product was purified by the reversed phase HPLC on a Nucleosil C_{18} column (4.6 × 250 mm, Supelco, United States) in the concentration gradient of acetonitrile in 0.1% TFA (pH 2.2) from 10 to 70% within 160 min at a flow rate of 0.5 mL/min.

Determination of the inhibition constants of the activity of the proteolytic enzymes. The HCGS 1.20 polypeptide in the concentration range from 0.008 to 7.0 μM was added to the 0.2 μM solution (10 μL) of trypsin/α-chymotrypsin in 50 mM Tris-HCl (pH 8.0). The reaction mixture was diluted with the buffer solution to the volume of 150 μL and incubated for 10 min at 37°C. The substrate solution (50 μL) in 50 mM Tris-HCl (pH 8.0) containing 10% DMF was added to the reaction mixture. The 0.6 mM *N*-benzoyl-*DL*-arginine *p*-nitroanilide hydrochloride (BAPNA) and 1.2 mM ethyl ester of *N*-benzoyl-*L*-tyrosine (BTEE) were used as substrates for trypsin and chymotrypsin in the concentrations of 0.2 and 0.3 mM, respectively. The constants were determined by the Dixon method [31] 30 min after the incubation at 37°C. The optical absorption of the formed *p*-nitroanilide or tyrosine was measured on an xMark flatbed rider (BioRad, United States) at 410 nm (for trypsin) or at 256 nm (for α-chymotrypsin), respectively. The constants were calculated on the basis of three parallel experiments, and the limits of the estimated error were 0.1–0.5%.

Mouse bone marrow-derived macrophages were obtained by the standard procedure [32]. Thigh bones of mice of the Balb/c line were separated under sterile conditions, placed in a Petri dish with the DMEM culture medium and put on ice. The bones were cleaned of muscle, epiphyses were cut off, and bone marrow was washed out from a medullary space by a syringe with the DMEM medium. The fragments of the bone marrow were resuspended by a pipet in the following culture medium: 50% DMEM medium with L-glutamine, 30% conditional DMEM medium which was prepared as a result of culturing of mouse L929 fibroblasts, and 20% horse blood serum. The cellular suspension was placed in nonadhesive plastic Petri dishes and incubated in the atmosphere of 5% CO_2 at 37°C for 7 days to the complete differentiation of the cells.

The content of the intracellular Ca^{2+} in the mouse bone marrow-derived macrophages was measured by the standard method with insignificant modification [33]. The culture of the bone marrow macrophages was washed with the phosphate buffer (pH 7.4). The cell (5×10^3 cells per one well) were washed out by an intensive pipet, placed in wells of a 96-well plate, kept for 2 h for adhesion, and washed with Hanks solution. The Fluo-3/AM fluorescent calcium-sensitive probe (100 μL/well, 5 μM) was added to the wells in the following buffer solution: 145 mM NaCl, 10 mM glucose, 5 mM KCl, 0.8 mM $MgCl_2$, 2 mM $CaCl_2$, and 10 mM HEPES (pH 7.4). The plate was incubated at 37°C for 40 min. The cellular monolayer was washed with the same medium three times, and 100 μL of the

medium were added in each well. The cells were kept at room temperature for 30 min. The base fluorescence was measured for 2 min. The histamine solution (20 μL, 50 μM) was added to the wells, and the change in the fluorescence intensity was registered over 1–3 min. The inhibiting effect of the HCGS 1.20 polypeptide (1, 10, and 100 μM) or fexofenadine (10 μM) was studied on the cells with the fluorescent probe which were preliminarily incubated with the examined compounds (10 μM) for 30 min. The fluorescence intensity was measured at $\lambda_{ex}/\lambda_{em}$ = 485 nm/520 nm on a PHERAstar FS microplate fluorescent photometer (BMG Labtech, Germany). The maximum intensity of the fluorescence of the control cells in the presence of histamine was taken to be 100%.

Determination of the content of the intracellular NO in the culture of the RAW 264.7 mouse macrophages. The cells of the RAW 264.7 line were cultured in the DMEM medium containing 10% embryonic calf serum in the atmosphere of 5% CO_2 at 37°C. The cells (5×10^3 cells/well) were placed in 96-well plates 2–3 h before the beginning of the experiment. When complete adhesion was achieved, the solutions of the HCGS 1.20 polypeptide (10 μM) and/or LPS (0.5 μg/mL) were added to the cells and incubated for 24 h. The FA-OMe fluorescent NO-sensitive probe [17] in a concentration of 10 μM was added to the cells in the course of 8 h for the determination of a level of the endogenously synthesized NO. The cellular monolayer was washed with the phosphate buffer (pH 7.4), and the fluorescence intensity was measured at $\lambda_{ex}/\lambda_{em}$= 485 nm/520 nm on a PHERAstar FS microplate fluorescent photometer (BMG Labtech, Germany). The maximum fluorescence intensity of the cells in the presence of LPS was taken to be 100%.

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