CLINICAL-DIAGNOSTIC STUDIES

THE STUDY OF BIODEGRADATION OF GALANIN AND ITS N-TERMINAL FRAGMENTS IN A MODEL SYSTEM *IN VITRO*

D.V. Avdeev[1]*, O.Yu. Selyutina[2], M.V. Sidorova[1], O.I. Pisarenko[1]

[1]Chazov National Medical Research Center for Cardiology,
15a Acad. Chazov str., Moscow, 121552 Russia; *e-mail: mityaavdeev93@mail.ru
[2]Voevodsky Institute of Chemical Kinetics and Combustion SB RAS,
3 Institustkaya str., Novosibirsk, 630090 Russia

Exogenous N-terminal fragments of galanin, which are agonists of the GalR2 receptor, have therapeutic potential in experimental cardiac pathology. This implies the need to study their proteolytic stability in biological environments. The aim of this work was to evaluate the proteolytic degradation of galanin G1 (GWTLNSAGYLLGPHAIDNHRSFSDKHGLT-NH_2), its natural and modified fragments G2 and G3 (WTLNSAGYLLGPHA-OH and WTLNSAGYLLGPβAH-OH, respectively) in human plasma. The peptides were obtained by solid-phase synthesis using the Fmoc methodology, purified by HPLC; their structure was confirmed by MALDI-TOF mass spectrometry and ^{1}H-NMR spectroscopy. The kinetics of galanins G1–G3 degradation in blood plasma was studied by ^{1}H-NMR spectroscopy based on changes in the intensity of Trp2 signals at 310 K. The results indicate a higher proteolytic stability of the G3 peptide compared to the natural G2 fragment and full-length galanin G1. They indicate the potential of using modified peptide agonists of GalR2 receptors to protect vital organs in pathophysiological conditions.

Keywords: galanin; N-terminal fragments of galanin; ^{1}H NMR; human plasma

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INTRODUCTION

The neuropeptide galanin (G1) is widely distributed in the central and peripheral nervous system, as well as in other tissues [1]. In peripheral organs, including the heart, G1 acts not only through neuronal mechanisms, but also by activating members of the GalR1-3 transmembrane receptor family [2]. G1 is involved in vital processes such as memory, food consumption, falling asleep, production of a number of hormones; at the cellular level it participates in maintaining ion homeostasis and osmosis [1]. The role of G1 receptors in the regulation of the cardiovascular system in various pathologies is still poorly understood. Recently, we have shown that intravenous administration of G1 (GWTLNSAGYLLGPHAIDNHRSFSDKHGLT-NH_2) to rats after regional myocardial ischemia reduces necrotic damage to cardiomyocytes [3]. This effect is mediated by activation of GalR2 receptors and is significantly reduced by the GalR2 antagonist M871 [4]. The N-terminal fragment of galanin (amino acid residues 2–15), WTLNSAGYLLGPHA-OH (G2), which exhibits a high affinity for the GalR2 receptor subtype, increases cell viability, inhibits apoptosis and the formation of superoxide and hydrogen peroxide in the mitochondria of H9C2 rat cardiomyoblasts during hypoxia/reoxygenation [5]. A chemically modified analogue of G2, the chimeric GalR2 receptor agonist WTLNSAGYLLGPβAH-OH (G3), reduces infarct size and the activity of necrosis markers in rat plasma during reperfusion [4]; it also reduces left ventricular dysfunction and improves the energy state and mitochondrial function in the heart of rats with doxorubicin cardiomyopathy [6]. The study of galanin receptor expression in the heart has shown that GalR2 is the dominant galanin receptor subtype in the myocardium, cardiomyocytes, and H9C2 cardiomyoblasts [7]. Genetic suppression of GalR2 promotes cardiac hypertrophy, fibrosis, and excessive generation of reactive oxygen species (ROS) in cardiac mitochondria [8]. These results demonstrate the possibility of correcting cardiac metabolism and function by using galanin and its high-affinity GalR2 receptor agonists G2 and G3 under conditions of energy deficiency and oxidative stress. Pharmacological use of these ligands requires studying their half-lives and degradation kinetics in plasma, since the available literature data are extremely limited. It is known that the half-life of G1 (1–29) *in vivo* is no more than 5 min [9, 10], and information on the proteolytic stability of G2 and G3 peptides is absent. In this regard, the aim of the present work was to study the proteolytic degradation of G1–G3 peptides in human plasma using ^{1}H-NMR spectroscopy.

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In this work, we report the identification of some degradation products of full-length galanin G1, its natural and modified fragments G2 and G3, and an assessment of their stability in an *in vitro* model system.

MATERIALS AND METHODS

Reagents

Fmoc-protected amino acid derivatives were purchased from Novabiochem (Switzerland) and Bachem (Switzerland). Reagents for peptide synthesis were purchased from Fluka Chemie GmbH (Switzerland). Solutions were prepared using deionized water (Millipore Corporation, USA). Deuterated water (SOLVEX, Russia, 99.8% D) was used to prepare samples for NMR experiments.

Synthesis of Galanin Peptides

Full-length rat galanin G1 was obtained by convergent solid-phase synthesis using the Fmoc methodology on the Rink amide polymer [11]. During the synthesis, the peptide chain was extended by one amino acid at a time and by fragment condensation. Fragments with a C-terminal glycine residue were obtained by a solid-phase method on 2-chlorotrityl chloride resin or in solution. Fragment condensation was carried out on a polymer carrier using complex F — adduct of N,N′-dicyclohexylcarbodiimide and pentafluorophenol (molar ratio of 1:3) [11]. Peptide G2 is the N-terminal fragment of the galanin sequence. Peptide G3 is a chimeric molecule, in which the C-terminal part of the galanin fragment (2–13), containing the pharmacophore amino acid residues Trp2, Asn5, Gly8, and Tyr9 [12] necessary for binding to cellular receptors GalR2, is supplemented with a sequence of the dipeptide carnosine βAla-His-OH. Carnosine is highly soluble in aqueous media and is known as a direct-acting antioxidant; it contains β-amino acid, which increases proteolytic stability of peptides [13]. Automated synthesis of peptides G2 and G3 [14] was carried out on a Tribute-UV synthesizer (ProteinTechnologies Inc., USA) on a 0.15 mmol scale in accordance with the single condensation program of Fmoc-amino acids (Novabiochem), using a polymer carrier with a 2-chlorotrityl chloride anchor group (Iris Biotech, Germany), containing 1.2 equiv. Cl/g. To create the amide bond, N,N,N′,N′-tetramethyl-O-(benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) was used in the presence of a twofold molar excess of N-methylmorpholine. Upon synthesis completion peptides G1–G3 were cleaved from the polymer with simultaneous removal of the protective groups by using a mixture TFA–water–triisopropylsilane–dithiothreitol (85 : 5 : 5 : 5). The solid-phase synthesis products were purified by reversed-phase high-performance liquid chromatography (HPLC) on a Knauer chromatograph with a column (250×20 mm) Eurospher 100-10 C18 (Knauer, Germany) to a purity of 97–98%. Buffer A (0.1% aqueous TFA solution) and buffer B (80% acetonitrile (Carl Roth GmbH, Germany) in buffer A) were used as eluents. Elution was performed from 100% buffer A with a linear gradient of buffer B concentration of 0.5%/min at a flow rate of 3 ml/min; detection was performed at 220 nm. Analytical HPLC was performed on a Smartline instrument (Knauer). HPLC conditions: Kromasil 100-5 C18 column (AkzoNobel, Sweden) (4.6×250 mm), buffer A — 0.1% aqueous TFA solution, buffer B — 80% acetonitrile in buffer A; elution at a rate of 1 ml/min with a concentration gradient of buffer B in buffer A from 20% to 80% in 30 min, detection at 220 nm. The structure of the peptides was confirmed using time-of-flight mass spectrometry (MS) with matrix-assisted laser desorption/ionization (MALDI-TOF). Mass spectra were recorded on a Bruker Autoflex speed mass spectrometer (Bruker Daltonics Inc., Germany). The structure of peptides G1–G3 was also confirmed by ^{1}H-NMR spectroscopy (spectrometer WH-500 Bruker 500 MHz, Germany). The amino acid sequences and characteristics of the peptides are presented in Table 1.

Human Plasma Preparation

Plasma was obtained from the blood of one healthy donor by venipuncture and sedimentation of cellular elements using a vacuum tube containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant, followed by centrifugation [15].

Table 1. Characteristics of galanin peptides

	Sequence	Molecular mass	MS (*m/z*)	HPLC		Solubility in water (mg/ml)
				R_t (min)	Purity (%)	
G1	GWTLNSAGYLLGPHAIDNHRSFSDKHGLT-NH_2	3164.45	3163.47 $[M]^+$	15.80	98.1	>40
G2	WTLNSAGYLLGPHA-OH	1499.67	1498.64 $[M]^+$	15.34	97.1	≈10
G3	WTLNSAGYLLGPβAH-OH	1499.67	1499.76 $[M]^+$ 1521.73 $[M+Na]^+$ 1537.72 $[M+K]^+$	14.66	98.2	>20

R_t – retention time.

Measurement of Galanin Peptide Concentrations in Plasma

To study the kinetics of galanin degradation in blood plasma, 450 µl-plasma aliquots were placed in a standard 5 mm NMR tube. 50 µl of D_2O was added to the samples for deuterium lock signal to achieve stabilization of the field frequency. The spectra were recorded with water signal suppression by means of gradient excitation (p3919gp pulse sequence). Difference spectroscopy was used to isolate signals belonging to peptide protons: the spectrum of plasma without the peptide was subtracted from each spectrum obtained after adding the peptide to the blood plasma. The experiments were carried out at 310 K. The total incubation time was 140 min. Signal assignment was done using spin decoupling technique. ^{1}H-NMR spectra were recorded on a Bruker Avance III 500 MHz spectrometer (Germany). The concentration of galanin peptides in the incubation medium was 2.5 mM. All experiments were repeated three times, the reaction rate constants are given as the mean ± standard deviation. Differences were considered statistically significant at $p<0.05$.

RESULTS AND DISCUSSION

To assess the stability of galanin peptides in human plasma, we used the ^{1}H NMR spectroscopy method, which allows monitoring peptide degradation without isolating degradation products from the incubation medium. We have previously used it for comparative assessment of the proteolytic stability of peptide inhibitors of myosin light chain kinase [15], apelin-12 and its modified analogue [16]. Figure 1 shows fragments of ^{1}H NMR spectra obtained during degradation of the G2 peptide in blood plasma.

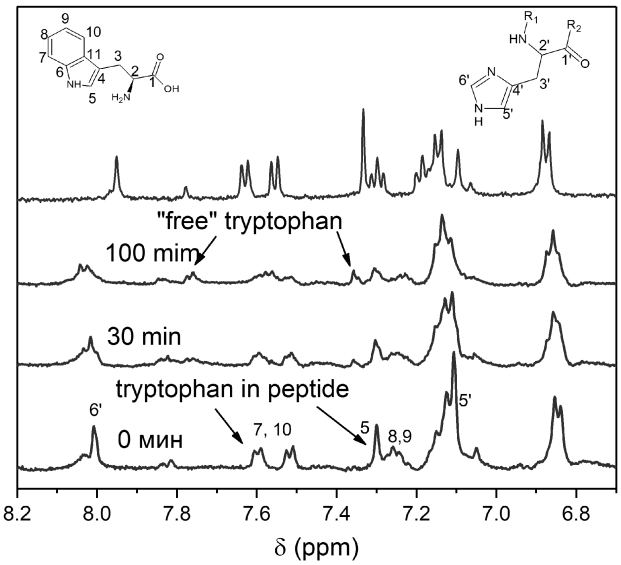

Figure 1. Fragments of ^{1}H-NMR spectra of peptide G2 sequentially from top to bottom: in phosphate-salt buffer, in human blood plasma after 100 min incubation, 30 min incubation, 0 min incubation.

As the reaction proceeded, a decrease in the integral intensity of signals from the Trp2 amino acid residue, the part of the peptide, was noted. The decrease in the intensity of signals at 8.01 ppm and 7.1 ppm, corresponding to the protons (6′) and (5′) of the imidazole ring of His14, was also observed (Fig. 1). No decrease in the intensity of signals from other amino acid residues was observed. Thus, it can be concluded that the degradation of G2 occurs through the simultaneous cleavage of Trp2 and His14 residues with the formation of the TLNSAGYLLGP fragment. The degradation of G3 occurs similarly, with formation of the TLNSAGYLLGPβA fragment. The G1 degradation presumably occurs with the formation of TLNSAGYLLGP and AIDNHRSFSDKHGLT-NH_2. Due to the absence of the key amino acid residues of G1 and Trp2, required for binding to galanin receptors [11], the degradation products of TLNSAGYLLGP and TLNSAGYLLGPβA should exhibit low affinity for GalR2 and weak bioactivity. This is due to the fact that the N-terminal end of the full-length galanin G1 is crucial for the biological activity of the peptide, while its C-terminal part is responsible for steric protection of the N-terminal part of the peptide from proteolytic degradation [17]. It is not associated with the GalR1-3 receptors and does not possess biological activity [18]. Probably, this can be fully attributed to another product of G1 degradation AIDNHRSFSDKHGLT-NH_2 caused by the His14 cleavage.

To analyze degradation kinetics of peptides G1–G3, the signals from the protons H-7 and H-10 of the indole ring of the Trp amino acid residues were selected (Fig. 1). The time dependences of the integral intensity of these signals are shown in Figure 2. It is evident that practically during the entire incubation, the intensity of the Trp proton signals decreased in the following order: G3 > G2 > G1. Table 2 shows the calculated rate constants of the Trp amino acid cleavage reaction and the half-lives of the peptides in plasma. The observed rate constants of G1 peptide degradation

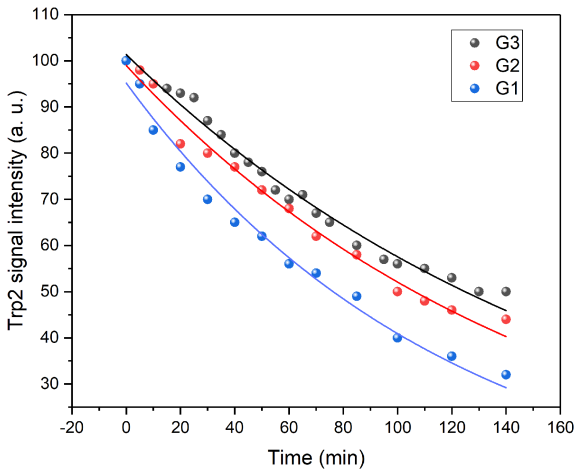

Figure 2. Kinetics of changes in the integrated signal intensity of Trp protons within galanin G1-G3 peptides.

Table 2. Observed rate constants for degradation of galanin peptides in plasma

Peptide	Observed reaction rate constant (k, s^{-1})	Half-life time, min
G1	$(14.1\pm0.7)\times10^{-5}$	76
G2	$(10.7\pm0.4)\times10^{-5}$*	106
G3	$(9.4\pm0.3)\times10^{-5\#}$	125

*p=0.014 versus G1; #p=0.004 versus G1.

were significantly higher than those of the N-terminal fragments of G2 and G3. Although the differences in this parameter between G2 and G3 peptides did not reach the level of statistical significance, there was a clear level of statistical tendency (p=0.06). According to these data, the shortest half-life was found in the full-length galanin G1, while in the G2 and G3 fragments it was 1.4 and 1.6 times longer, respectively. It should be noted that one of the limitations of the approach used in this work to assess the proteolytic stability of galanin peptides is the relatively low sensitivity of the ^{1}H-NMR method. This leads to the need to work in the range of galanin concentrations significantly exceeding the pharmacological ones. Therefore, the half-life of the G1 peptide in our experiments is higher than the values obtained in plasma in *in vivo* models [9, 10]. Despite this, we had the opportunity to compare the stability of the structure in a number of peptide analogues under the same conditions, regardless of the type of laboratory animals. The main result of the work is the detection of a higher proteolytic stability of the chimeric agonist of GalR2 receptors peptide G3 compared to the natural fragment G2 and full-length galanin G1.

Recent studies have revealed the role of galanin and its natural and modified N-terminal fragments in the regulation of the metabolic and functional state of the heart in various forms of cardiovascular stress [19–22]. Analysis of galanin receptor mRNA levels indicates that GalR2 predominates in cultured myocytes and the heart [7]. Moreover, analysis of galanin receptors by means of specific antibodies and immunofluorescence microscopy, revealed that their expression in the heart decreases in the following order: GalR2 > GalR1 > GalR3 [23]. These data suggest a key role of GalR2 receptors in their action on the myocardium. We have previously demonstrated that a pharmacological G3 agonist exerts a protective effect in ischemic/reperfusion injury of the heart [19], doxorubicin-induced cardiomyopathy [6], and streptozotocin-induced diabetes [24]. Taken together, these experimental facts indicate the fundamental role of GalR2 receptor activation in the realization of the effects of galanin peptides and suggest the feasibility of molecular design of pharmacological agonists with improved physicochemical characteristics (proteolytic stability and solubility). Such synthetic peptide bioregulators can be used to develop drugs that reduce stress-induced changes in the body and heart.

CONCLUSIONS

The degradation kinetics of GWTLNSAGYLLGPHAIDNHRSFSDKHGLT-NH_2 (G1), the natural N-terminal fragment of galanin WTLNSAGYLLGPHA-OH (G2), and its modified analogue WTLNSAGYLLGPβAH-OH (G3) in human plasma has been studied using ^{1}H-NMR spectroscopy. The half-life of galanin peptides increased in the following order: G1 < G2 < G3. We believe that the creation of proteolytically stable peptide agonists of galanin receptors GalR2 is a promising approach to activating the galaninergic system to protect vital organs in pathophysiological conditions.

FUNDING

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COMPLIANCE WITH ETHICAL STANDARDS

The blood collection procedure performed by volunteers complied with the ethical standards of the National Research Committee, as well as the 1964 Helsinki Declaration and its later amendments. Informed consent was obtained from the volunteer before participation in the study.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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ИЗУЧЕНИЕ БИОДЕГРАДАЦИИ ГАЛАНИНА И ЕГО N-КОНЦЕВЫХ ФРАГМЕНТОВ В МОДЕЛЬНОЙ СИСТЕМЕ *IN VITRO*

Д.В. Авдеев[1]*, О.Ю. Селютина[2], М.В. Сидорова[1], О.И. Писаренко[1]

[1]Национальный медицинский исследовательский центр кардиологии им. ак. Е.И. Чазова,
121552, Москва, ул. Академика Чазова, 15а; * эл. почта: mityaavdeev93@mail.ru
[2]Институт химической кинетики и горения им. В.В. Воеводского
Сибирского отделения Российской академии наук, Новосибирск, 630090, ул. Институтская, 3

Экзогенные N-концевые фрагменты галанина, являющиеся агонистами рецептора GalR2, обладают терапевтическим потенциалом при экспериментальной патологии сердца, что предполагает необходимость изучения их протеолитической устойчивости в биологических средах. Цель настоящей работы состояла в оценке протеолитической деградации галанина G1 (GWTLNSAGYLLGPHAIDNHRSFSDKHGLT-NH_2), его природного и модифицированного фрагментов G2 и G3 (WTLNSAGYLLGPHA-OH и WTLNSAGYLLGPβAH-OH, соотв.) в плазме крови человека. Пептиды были получены твердофазным синтезом с использованием Fmoc-методологии, очищены с помощью ВЭЖХ, их структура подтверждена методами MALDI-TOF масс-спектрометрии и ^{1}H-ЯМР спектроскопии. Кинетику деградации галанинов G1–G3 в плазме крови изучали методом ^{1}H-ЯМР спектроскопии по изменению интенсивности сигналов от Trp2 при температуре 310 K. Результаты свидетельствуют о большей протеолитической стабильности пептида G3 по сравнению с природным фрагментом G2 и полноразмерным галанином G1. Они указывают на перспективность использования модифицированных пептидных агонистов рецепторов GalR2 для защиты жизненно важных органов при патофизиологических состояниях.

Полный текст статьи на русском языке доступен на сайте журнала (http://pbmc.ibmc.msk.ru).

Ключевые слова: галанин; N-концевые фрагменты галанина; ^{1}H ЯМР; плазма человека

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