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Protective Effects of a Novel Agonist of Galanin Receptors Against Doxorubicin-Induced Cardiotoxicity in Rats

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Abstract

The clinical use of antineoplastic agent doxorubicin (DOX) is limited due to its cardiotoxic action. [βAla14, His15]-galanine (2–15) (G) is a novel synthetic agonist of galanin receptors GalR1-3 having cardioprotective properties in animal models in vivo. The aim of the present study was to explore effects of G on DOX-induced cardiotoxicity. Wistar rats were divided into four groups and treated with DOX (D group), DOX and G (D + G group), G (G group), and saline (control). Before treatment and at the end of the study, concentration of thiobarbituric acid reactive substances (TBARS) and activity of creatine kinase-MB (CK-MB) were determined in blood plasma, the animals were weighed, and cardiac function was evaluated by echocardiography. At the end of experiments, the hearts were used to determine energy metabolites and mitochondrial respiration in permeabilized fibers. After an 8-week study, D group exhibited a pronounced cardiac failure, the absence of weight gain, an increased plasma TBARS concentration, and CK-MB activity. These disorders were accompanied by a reduced myocardial content of high-energy phosphates and mitochondrial respiratory parameters. Co-administration of G with DOX significantly decreased plasma TBARS level and prevented an increase in plasma CK-MB activity. In D + G group, myocardial contents of ATP, PCr, total adenine nucleotides, and total creatine as well as myocardial PCr/ATP ratio and the respiratory control index were higher than in D group at the end of the experiments. Peptide G significantly improved parameters of left ventricular (LV) function and caused weight gain in animals of D + G group. These results suggest that peptide G may be a potential pharmacological agent that attenuates the cardiotoxic effects of DOX.

Keywords Doxorubicin · Cardiotoxicity · Galanin receptor agonist · Rats · Cardiac function · TBARS · Myocardial bioenergetics

Introduction

Anthracycline antibiotic doxorubicin (DOX) is one of the most effective chemotherapeutic agents used in a broad spectrum of malignancies due to its high antineoplastic efficacy [1]. Despite its wide use, DOX has severe side

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effects with cardiotoxicity being the most important [2]. DOX cardiotoxicity is a cumulative and dose-dependent progressive myocardial lesion ranging from asymptomatic reduction in the left ventricular ejection fraction (LVEF) to irreversible life-threatening congestive heart failure [3]. In recent decades, attempts have been made to obtain less cardiotoxic anthracyclines (for example, epirubicin or idarubicin). However, these drugs have less antitumor activity and are capable of causing myocardial injury immediately after infusion [4]. Other approaches to limit anthracycline-induced cardiotoxicity included slow infusions, the combined use of iron chelators and antioxidants, or the treatment with liposomal-encapsulated doxothracyclines [5–8]. It is recognized that these strategies provide only a limited improvement in cardiac function. So far, DOX continues to be the main drug for the treatment of various human tumors, and its cardiotoxicity remains a clinical dilemma [4]. Multiple mechanisms are involved in DOX-induced heart failure. One of the main hypotheses explaining the pathophysiological effects of DOX is that cardiac damage is caused by oxidative stress through the generation of reactive oxygen species (ROS) [9, 10]. Topoisomerase IIβ (TOP2B) is involved in DOX-induced DNA double-strand breaks and transcriptome changes that are responsible for mitochondrial biogenesis disorders and ROS formation [11, 12]. In particular, it was shown that the bisdioxopiperazine dexrazoxane (DZR) reduces the incidence of anthracycline-induced heart failure via TOP2B degradation in cardiomyocytes [13]. Mitochondria are a primary target of DOX-induced cardiotoxicity mediated by ROS formation [14]. Both acute and chronic DOX administrations lead to a dramatic diminution of mitochondrial function revealed by a decrease in respiration and a reduction in myocardial high-energy phosphates [15, 16]. Cytosolic and mitochondrial isoforms of creatine kinase (CK) are also vulnerable to ROS-mediated damage and an increase in ferrous ions when doxorubicin is administered [17]. Thus, DOX simultaneously violates the formation of energy and its transfer to myofibrils. Most studies show that along with the increased ROS production subsequent apoptosis is the pivotal adverse factor in the pathogenesis of DOX-induced cardiomyopathy [14]. Not only cardiomyocytes but also endothelial cells are affected by DOX-induced apoptosis in vivo, as indicated by caspase activation and internucleosomal DNA degradation [18]. At the cellular level, activation of lipid peroxidation caused by ROS formation results in disruption of the cardiac myocyte structure, including damage to the microtubules, sarcomere disruption, and loss of myofibrils [19]. In addition, a decreased activity of antioxidant enzymes and altered intracellular calcium homeostasis, which are initiated by ROS, have been proposed to explain cardiotoxicity of DOX [9, 14]. These experimental facts suggest that compounds acting as radical scavengers, iron-chelating agents, and metabolic protectors can be useful to reduce DOX-induced cardiac damage.

Nowadays, targeting galanin receptors is promising for the treatment of various human pathological conditions, including Alzheimer's disease, metabolic diseases, solid tumors, and ischemic heart disorders [20]. We have recently shown that natural and modified N-terminal fragments of galanin (2–11) and (2–15) reduce experimental myocardial I/R injury [21, 22]. They are able to increase cell viability and inhibit apoptosis and mitochondrial ROS formation in rat H9c2 cardiomyoblast cells subjected to hypoxia-reoxygenation [21, 23]. Postischemic infusion of these peptides improves functional recovery of isolated rat heart during reperfusion, reduces cell membrane damage, and enhances restoration of myocardial energy state. Intravenous administration of the peptides limits myocardial infarct size and decreases activity of necrosis markers in rats in vivo [21–23]. The beneficial effects of these peptide ligands are probably associated with activation of the GalR2 receptor, since they have a poor affinity for the receptor subtypes GalR1 and GalR3 [24]. Based on the above facts, we have designed the present study to evaluate effects of the most efficient chimeric ligand [βAla14, His15]-galanine (2–15) (G) on DOX-induced cardiotoxicity in rats. Earlier the action of peptide G on animals treated with DOX and underlying mechanisms have not been studied. Our research focuses on the parameters of LV cardiac function, oxidative stress, and myocardial energy state. The results demonstrate that treatment with peptide G increases functional and metabolic tolerance of the heart to oxidative stress caused by DOX administration.

Methods

Synthesis of Peptide G

Peptide G, the modified galanin fragment (2–15) WTLNSAGYLLGPβAH, was synthesized by method of Fmoc (9-fluoroenylmethyloxycarbonyl) solid phase peptide synthesis (SPPS) in stepwise automatic mode using peptide synthesizer Tribute-UV (Protein Technologies Inc., USA). Amino acid side chain functional groups were blocked with acid labile protecting groups designed for Fmoc strategy: *tert*-butoxycarbonyl (Boc) at indole moiety of Trp; *tert*-butyl (Bu^t) at hydroxyl groups of Ser, Thr and Tyr; trityl (Trt) at imidazole ring of His and at carboxamide function of Asn. We used preloaded Fmoc-His(Trt)—Wang resin (Iris Biotech GmBH, Germany) with substitution 0.51 mmol/g in scale 0.5 mmol of amino acids. Cleavage of the Fmoc protecting group was achieved using 25% 4-methylpiperidine in *N,N*-dimethylformamide

(DMF) 2 × 5 min. Amino acids were coupled using 4 equiv amino acid, 4 equiv 2-(1H-benzotriazol-1-yl)-*N,N,N′,N′*-1,1,3,3-tetramethylammonium tetrafluoroborate (TBTU), and 8 equiv *N*-methylmorpholine in DMF. Peptide was completely deprotected using trifluoroacetic acid (TFA)/H_2O/triisopropylsilane/dithiothreitol (85:5:5:5) for 1.5 h at room temperature.

Crude peptide contained 82% of the basic substance according to high-performance liquid chromatography (HPLC). The crude product of SPPS was purified by preparative HPLC performed on a Knauer (Germany) system using Eurosphere columns (20 × 250 mm^2, 10 µm) (Knauer, Germany). The elution was achieved with a linear gradient (0.5% /min) of aqueous 0.01M NH_4OAc, pH 4.5 (A) and 80% acetonitrile in water (B) at a flow rate of 10 ml/min with UV detection at $\lambda = 220$ nm. After purification and lyophilization, substance was 98% of homogeneity.

Structure of chimeric peptide G was confirmed by both proton nuclear magnetic resonance (^{1}H-NMR) and mass spectrometry techniques. ^{1}H-NMR spectra were obtained using a WM-500 spectrometer (Bruker, Germany) in DMSO-d_6 at 300 K. Concentration of peptide was 2–3 mg/ml. Chemical shifts were registered relative tetramethylsilane. Mass spectrometry data were obtained using an Ultrafex matrix-assisted laser desorption and ionization time of flight mass spectrometry instrument (MALDI-TOF, Bruker Daltonics, Germany). Peptide G characteristics are shown in Table 1.

Chemicals

Enzymes and chemicals were purchased from Fluka (Switzerland) and Sigma Chemical Co. (St. Louis, MO USA). Solutions were prepared using deionized water (Millipore Corp. Bedford, MA, USA). Doxorubicin hydrochloride was purchased from Merck (Germany).

Animals

Forty-eight male Wistar rats weighing 300–340 g were housed in cages in groups of three, maintained at 20–30 °C with a natural light–dark cycle. All animals had free access to standard pelleted diet (Aller Petfood, St. Petersburg, Russia) and tap water. Principles of laboratory animal care were followed during this study in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). The experimental protocol was approved by the local Institutional Animal Care Committee.

Experimental Study Design

In this study, a regimen of subclinical DOX-induced cardiotoxicity by a modified Kang method was used [25]. Rats were randomized into four groups with 12 rats each: the control group (C) received saline weekly (1.0 ml/kg for 8 weeks, subcutaneously); the DOX group (D) received DOX (2.0 mg/kg/week for the first 4 weeks, intraperitoneally) and then saline (1.0 ml/kg/week for the next 4 weeks, subcutaneously); the DOX + peptide G group (D + G) received DOX and saline as stated above and simultaneously peptide G (50 nmol/kg/day for eight weeks, subcutaneously); the peptide G group (G) received peptide G (50 nmol/kg/day for 8 weeks, subcutaneously). The dose of peptide G was chosen based on preliminary experiments. The applied DOX dose (2 mg/kg) corresponds to a human equivalent dose (HED) of about 13 mg/m^2 [26]. However, this conversion between species is not always supported for drugs administered by an intraperitoneal route. We used a subcutaneous route of peptide G administration since it provides a sustained effect and allows injecting smaller volumes minimizing adverse effects [27]. This method of peptide G administration did not cause skin irritation or anxiety. Immediately before treatment with drugs and the next day after an 8-week study, blood samples were collected from the tail vein for determination of plasma thiobarbituric acid reactive substances (TBARS) and activity of creatine kinase-MB (CK-MB), the animals were weighed,

Table 1 Physico-chemical characteristics of a modified galanin fragment G

Peptide	Sequence	MW, g/mol	MALDI-TOF (m/z)	HPLC[a] R_t (min)	Yield (%)[b]	SW (mg/ml)
G	H-WTLNSAGYLLGP**βAlaH-**OH	1499.67	1499.76	14.66	46.3	20.0

Carnosine part is shown in bold

R_t retention time, *SW* solubility in water

[a]Analytical HPLC was performed on a Gilson chromatograph (France) on Kromasil 100 C18 columns (5 µm, 4.6 × 250 mm) (Sweden) using gradient (20–80%) of concentrations of buffer B (80% acetonitrile and 0.1% TFA) in buffer A (0.1% TFA) for 30 min at a flow rate of 1 ml/min and detection at 220 nm

[b]The yield is given relatively to the first amino acid attached to the polymer

and cardiac function was assessed. At the end of experiments, anesthetized rats (urethane, 120 mg/kg body wt intraperitoneally) with the chest opened by a left thoracotomy in the fifth intercostal space were artificially ventilated with a KTR-5 animal respirator (Hugo Sachs Electronik GmbH) to excise beating hearts for the subsequent evaluation of myocardial energy metabolites and mitochondrial respiration in permeabilized fibers.

Myocardial Metabolites

Six hearts from each group ($n = 6$) were frozen in liquid nitrogen using a Wollenberger clamp. The frozen hearts were quickly homogenized in cooled 6% $HClO_4$ (10 ml/g) using an Ultra-Turrax T-25 homogenizer (IKA-Labortechnik, Staufen, Germany), and the homogenates were centrifuged at 2800×*g* for 10 min at 4 °C. The supernatants were neutralized with 5 M K_2CO_3 to pH 7.40, and the extracts were centrifuged after cooling to remove $KClO_4$ precipitate. Tissue dry weights were determined by weighing a portion of the pellets after extraction with 6% $HClO_4$ and drying overnight at 110 °C. Concentrations of ATP, ADP, AMP, phosphocreatine (PCr), creatine (Cr), and lactate in neutralized tissue extracts were determined by enzymatic methods [28].

Permeabilized Myocardial Fibers

The remaining six hearts of each group were used to prepare permeabilized fibers according the method described in [29]. In brief, muscle strips (2–4 mm long and 1–1.5 mm in diameter, 5–7 mg of wet weight) were cut from endocardium of the LV. The muscle fibers were separated from each other by using sharpened forceps in cooled (in ice) solution A. Solution A contained, in mM, CaK_2EGTA 2.77, K_2EGTA 7.23, $MgCl_2$ 6.56, dithiothreitol 0.5, K-MES 50, imidazole 20, taurine 20, Na_2ATP 5.3, phosphocreatine 15; pH 7.1 adjusted at 25 °C. The fiber bundles were transferred to a vial containing 2 ml of solution A and 50 µg/ml saponin and gently mixed at 4 °C for 20 min. The permeabilized fibers were then washed two times in the sterilized medium B for 10 min to remove ATP and saponin. Solution B consisted of EGTA 0.5 mM, $MgCl_2.6H_2O$ 3 mM, taurine 20 mM, KH_2PO_4 10 mM, HEPES 20 mM, BSA 1 g/liter, potassium-lactobionate 60 mM, mannitol 110 mM, dithiothreitol 0.3 mM; pH 7.1 at 25 °C. 2 ml of medium B and permeabilized fiber bundles (2–5 mg wet weight) were added to the oxygraph chamber (Oxygraph plus system, Hansatech Instr., UK) and equilibrated with atmospheric oxygen at experimental temperature of 37 °C. The energy substrates were 10 mM glutamate + 5 mM malate. Respiratory parameters of permeabilized myocardial fibers were measured after addition of 2 mM ADP and expressed in nmol O_2/min/mg dry weight. Dry weight of the muscle samples was determined after drying for 48 h at 80 °C.

Lipid Peroxidation Products and CK-MB Activity

Cayman's TBARS Kit was used for colorimetric measurement of lipid peroxidation products in plasma. Plasma CK-MB activity was determined by an immunoinhibition method using standard kits from BioSystems S.A. (Barcelona, Spain) from the rate of NADPH formation in the hexokinase and glucose-6-phosphate dehydrogenase coupled reactions.

Echocardiographic Study

Animals were anesthetized with an intraperitoneal injection of ketamine (50 mg/kg) and xylazine (1 mg/kg). The chest was shaved, and the animals were positioned on their left side. Echocardiography was performed using a Philips Envisor C ultrasound system with a 10 MHz electronic transducer. All parameters were obtained from parasternal long and short axis and apical four chambers. Image depth was 3–4 cm with 256 frames per second acquisition. Images were obtained from the left parasternal short-axis views of the left ventricle (LV) at the level of papillary muscles to define wall thicknesses and internal diameters during systole and diastole and regional wall motion abnormality in the LAD area. Left ventricular end-diastolic diameter (LVEDD) and left ventricular end-systolic diameter (LVESD) were measured from M-mode images. Left ventricular fractional shortening (FS) was calculated as (LVEDD-LVESD)/LVEDD × 100%. Left ventricular ejection fraction (EF) was calculated using the Teichholz method. Heart rate was determined from the distance between two consecutive cardiac cycles; all values were obtained from the average of three consecutive beats.

Statistical Analysis

Data are presented as means ± SEM. Results were analyzed by one-way ANOVA followed by Bonferroni multiple range test post hoc analysis for calculation differences between more than two groups. Comparisons between two groups involved use of the Student's unpaired *t* test. All statistical analyses were performed using SigmaPlot version 12 (Systat Software Inc, San Jose, CA). $P < 0.05$ was defined as significant. The data and statistical analysis complied with the recommendations on experimental design and analysis in pharmacology [30].

Results

Echocardiography

Effects of peptide G and DOX on cardiac function of rats were assessed by echocardiographic parameters. There were no significant differences in LVESD, LVEDD, FS, or EF between the groups before treatment. By the end of the eighth week, the DOX-treated group exhibited a pronounced cardiac dysfunction (Fig. 1A–D). A significant increase in LVEDD and LVESD combined with a reduction in FS and EF was detected in the D group compared with these indices in control. The cardioprotective effect of peptide G against LV systolic dysfunction was observed in the D + G group. It was manifested in a significant decrease in LVESD and LVEDD to the values not significantly different from those in the control group. Co-administration of peptide G and DOX significantly increased FS and EF compared with these parameters in the D group. After treatment of animals with G alone for 8 weeks, the echocardiography variables did not differ from those in the initial state and in the control group. In the G group, LVESD was significantly lower and both FS and EF were significantly higher than these indices in the D + G group.

Plasma TBARS Concentrations

Changes in oxidative stress biomarkers in the blood of animals are a characteristic feature of DOX-induced cardiotoxicity [31]. In the present study, we have evaluated effects of peptide G on plasma concentrations of TBARS, low molecular weight end products of oxidative damage. There were no significant differences in plasma TBARS levels between the groups before treatment. In the initial state, the average TBARS concentration in blood plasma 3.21 ± 0.14 μM was observed (Fig. 2A). After 8 weeks of the study, plasma TBARS concentration did not change in rats of the control group compared to this value. On the contrary, this index increased sevenfold in rats of the D group thus indicating activation of lipid peroxidation. Plasma TBARS

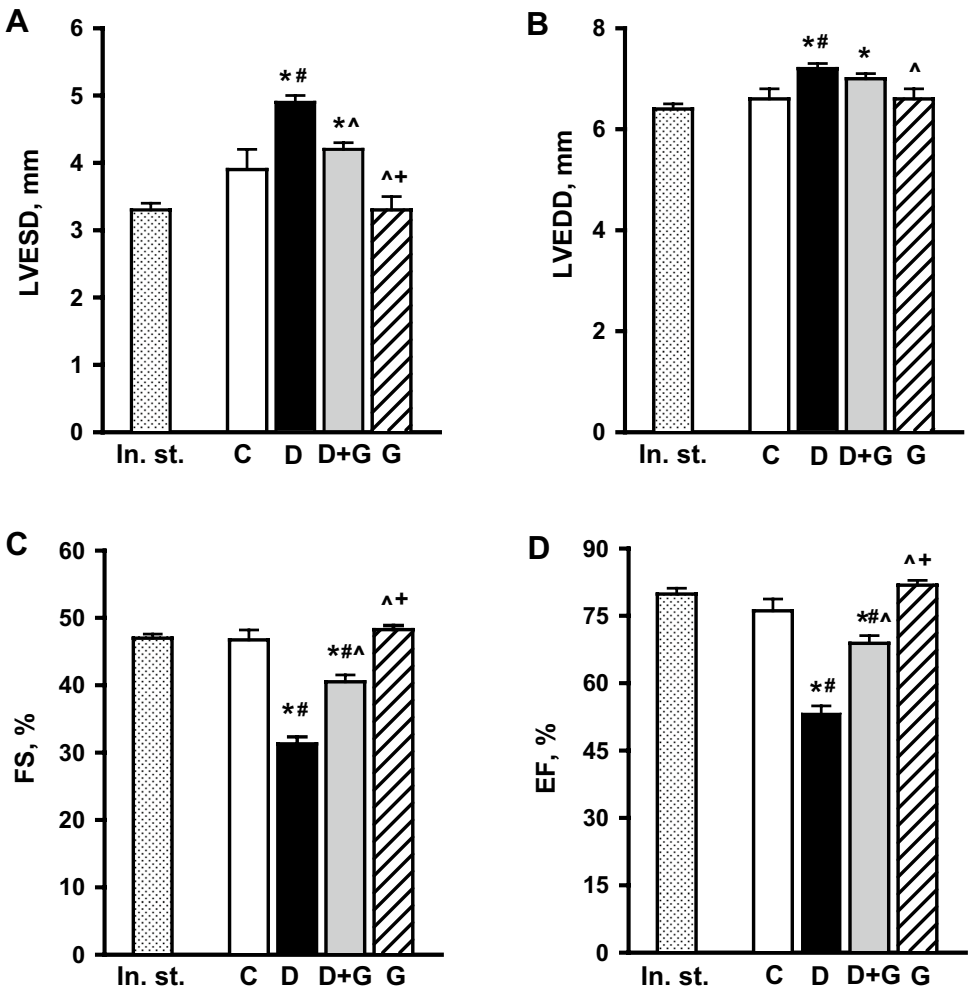

Fig. 1 Changes in echocardiographic variables during the study. In. st., initial state before treatment (the values correspond to the means + SEM for all groups, $n = 48$). *C* the control group, *D* the DOX group, *D* + *G* the DOX + peptide G group, *G* the peptide G group. **A** LVESD, left ventricular end-systolic diameter, **B** LVEDD, left ventricular end-diastolic diameter, **C** FS, LV fractional shortening, **D** EF, LV ejection fraction. The values for the C, D, D + G, and G groups correspond to the 8-week study and are expressed as means ± SEM for 12 animals. $P < 0.05$ versus *initial state, $^{\#}$C, $^{\wedge}$D, $^{+}$D + G

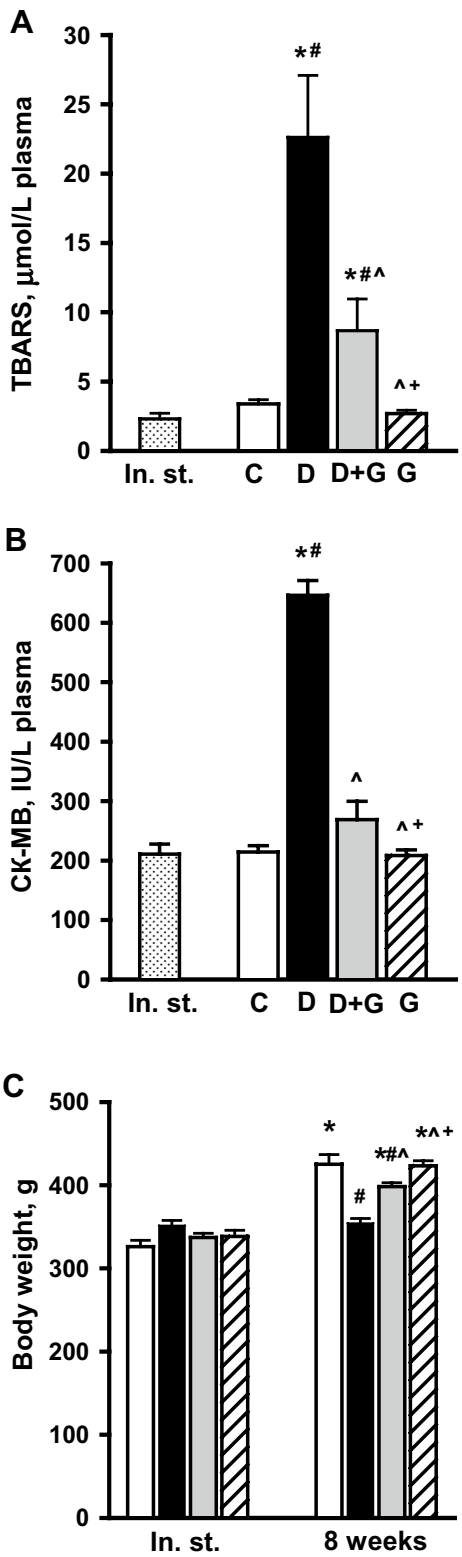

Fig. 2 Changes in plasma concentration of thiobarbituric acid reactive substances (TBARS) (**A**), plasma activity of creatine kinase-MB (**B**) and body mass of rats (**C**) of the control and treated groups during the study. (**A**) TBARS concentrations (in µmol/l plasma) are expressed as means ± SEM. In. st., initial state before treatment ($n = 24$), *C* the control group, D DOX group; *D* + *G* DOX + peptide G group, *G* peptide G group ($n = 6$). TBARS values for the C, D, D + G and G groups correspond to 8 weeks of the study for six animals in a group. $P < 0.05$ versus *initial state, $^{\#}$C, $^{\wedge}$D, $^{+}$D + G. **B** Activity of creatine kinase-MB (CK-MB) (in IU/l plasma) is expressed as mean ± SEM. In. st., initial state before treatment ($n = 24$), **C** the control group; **D** DOX group; D + G, DOX + peptide G group; G, peptide G group ($n = 6$). CK-MB activity for the C, D, D + G, and G groups correspond to 8 weeks of the study for 6 animals in a group. $P < 0.05$ versus *initial state, $^{\#}$C, $^{\wedge}$D, $^{+}$D + G. (C) Body mass of rats (in g) are expressed as means ± SEM for groups of 12 animals each. In. st., initial state before treatment; 8 weeks, after the 8-week study. $P < 0.05$ versus *initial state, $^{\#}$C, $^{\wedge}$D, $^{+}$D + G. Indication of the C, D, D + G, and G groups is the same as in (**A**)

concentration was 2.6 times lower in the D + G group than in the D group but remained significantly higher than in the control group. As expected, treatment of animals with peptide G for 8 weeks did not affect plasma TBARS level in comparison with the values in the initial state and in the control group.

Plasma CK-MB Activity

There were no significant differences in plasma CK-MB activity between the groups before treatment; the average initial activity of this cardiac marker was 211.0 ± 15.8 IU/l (Fig. 2B). After 8 weeks of the study, plasma CK-MB activity did not change in rats of the control group compared to this value. Treatment with DOX produced severe cardiotoxicity which was manifested in a threefold increase in CK-MB activity compared with the control. Plasma CK-MB activity was 2.4 times lower in the D + G group than in the D group (268.9 ± 30.8 and 646.2 ± 28.4 IU/l, respectively, $P < 0.001$) and did not differ from this index in the control group. These data suggest that peptide G significantly reduced the myocardial tissue injury caused by DOX injections. Treatment of rats with peptide G for 8 weeks did not affect plasma CK-MB activity in comparison with the initial state and the value in the control group. In the G group, plasma CK-MB activity was significantly lower than in the D + G group.

Body Mass

We have assessed the effect of peptide G on one of the most important markers of DOX toxicity, body mass loss [32]. There were no significant differences in the initial body mass between the groups (Fig. 2C). The control group demonstrated a progressive increase in body mass during the observation period. Throughout the study, animals of DOX group showed a pronounced impairment in growth. After 8 weeks of the study, the mean body mass in this group was the same as at the beginning of the study and was 20% lower than in the animals of the control group. Co-administration of G and DOX increased body mass during the study. By the end

of 8 weeks, body mass in the D + G group was significantly higher than in the D group, although it remained reduced compared to the control group. Throughout the observation period, the increase in body mass of animals treated with the peptide G was the same as in control animals. In the G group, this parameter was significantly higher than in the group D + G.

Myocardial Energetics

Myocardial energy status is a critical aspect of cardiac function. At the next stage, we examined effect of peptide G on metabolic support of the failing heart in DOX-treated rats. Myocardial contents of high-energy phosphates, respiratory control index (RCI) in permeabilized fibers characterizing oxidative phosphorylation, and myocardial PCr/ATP ratio reflecting development of LV dysfunction were used as criteria for the energy supply of the heart. After an 8-week study, the values of myocardial energetics indices in rats of the control group did not differ from those for intact animals that did not receive any drugs or saline [33] (Fig. 3A–F). In the D group, myocardial ATP was significantly reduced on average by 20% in comparison with this value in the control group. This resulted in a clear trend to a decrease in the total adenine nucleotide pool (ΣAN) in the hearts of DOX-treated rats since ADP and AMP levels in groups C and D did not differ significantly. Myocardial PCr and the total creatine (ΣCr = PCr + Cr) were 50 and 75%, respectively, of the values in the control group. The hearts of DOX-treated animals showed a twice lower myocardial PCr/ATP ratio and 1.6-fold decrease (although not statistically significant) in RCI as compared with the control. These data suggested impaired mitochondrial function, compromised cardiac energy homeostasis, and loss of sarcolemma integrity in animals that received DOX. Co-administration of DOX with peptide G attenuated the disturbances of myocardial energy metabolism. At the end of the experiments, the group D + G showed a trend towards an increase in myocardial ATP and PCr combined with a significant increase in both ΣAN and ΣCr pools. Noteworthy, in the hearts of

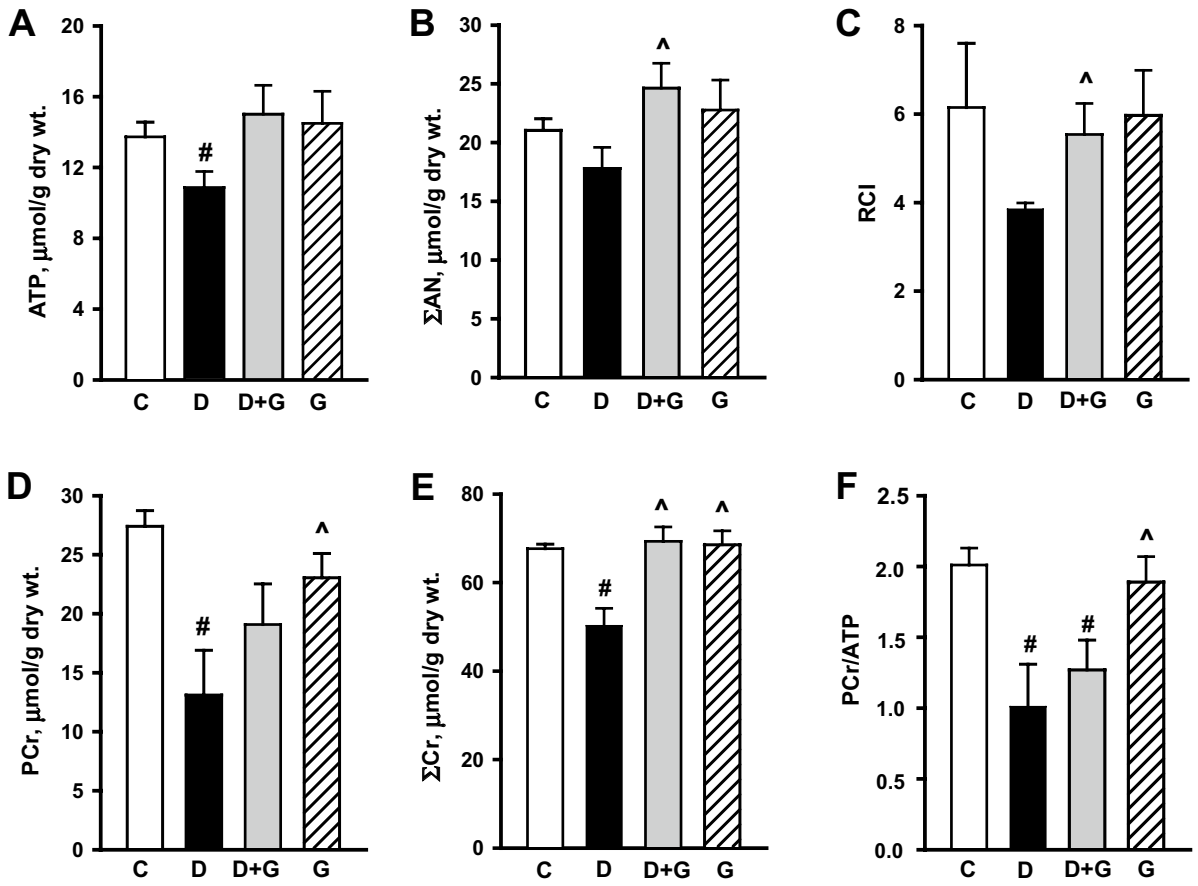

Fig. 3 Myocardial energy state in the control and treated groups after 8 weeks of the study. Values are expressed as means ± SEM for groups of 6 animals each. *C* the control group, *D* DOX group, *D + G* DOX + peptide G group, *G* peptide G group. Myocardial content of metabolites: **A** ATP, **B** ΣAN = ATP + ADP + AMP, **D** PCr, phosphocreatine, **E** ΣCr = phosphocreatine + creatine. **C** *RCI* respiratory control index. **F** PCr/ATP, myocardial PCr/ATP ratio. $P < 0.05$ versus $^{\#}$C, $^{\wedge}$D

animals of the group D + G, myocardial contents of ATP, PCr, ΣAN, and ΣCr as well as the adenylate energy charge (AEC = ATP + 0.5ADP/ΣAN) did not differ from the values in the control. In parallel, there was a slight increase in myocardial PCr/ATP ratio and a significant augmentation of RCI (by 25 and 43%, respectively) compared to those in the group D. Administration of peptide G for 8 weeks did not affect indices of myocardial energy metabolism and mitochondrial respiratory parameters of permeabilized fibers compared to the control group. In the G group, myocardial contents of PCr, ΣCr, and PCr/ATP ratio were significantly higher than in the DOX-treated group.

Discussion

DOX is a potent chemotherapeutic agent widely used in the treatment of various cancers. The clinical efficacy of DOX is severely limited by its cardiotoxicity, which leads to irreversible degenerative cardiomyopathy and heart failure [2–4]. Our study demonstrated for the first time that peptide G, a novel pharmacologic ligand of galanin receptors, is able to reduce the negative effects of DOX-induced chronic cardiotoxicity. It is known that DOX enters into cardiomyocytes by passive diffusion and stimulates the formation of ROS that can damage cells by lipid peroxidation. The DOX-induced oxidative stress causes mitochondrial dysfunction leading to a deficiency in energy production and irreversible damage to cardiomyocytes. The results of recent studies show that a DNA-binding enzyme topoisomerase 2 (TOP2) is the essential molecular driver of damaging effects of anthracyclines. By binding both DNA and TOP2, DOX forms a ternary TOP2–DOX–DNA cleavage complex, which in turn triggers cell death [11, 12]. These factors are largely responsible for the development of LV dysfunction [9–11]. In our model, toxicity caused by DOX was manifested as body mass loss associated with cardiac failure evidenced by a significant reduction in EF and FS, and enlargement in the LV cavity that was accompanied by a significant increase in plasma TBARS concentration and plasma activity of CK-MB. Co-administration of peptide G with DOX significantly reduced oxidative stress, which affected the decrease in TBARS level and weight gain. Treatment with peptide G prevented damage to cell membranes in the myocardium of animals receiving DOX as evidenced by lack of increase in plasma activity of CK-MB. In accordance with this, peptide G exhibited protective effects against LV dysfunction caused by DOX. Indeed, echocardiography study of animals treated with peptide G and DOX revealed significant improvement in both EF and EF, and LV diastolic and systolic diameters, which represented the enhancement of cardiac systolic function.

An interesting finding of this work was the effect of peptide G on myocardial energetics in DOX-treated animals. Impact of chronic DOX administration included a compromised mitochondrial function estimated by RCI and a decrease in the generation of high-energy phosphates with a reduction in myocardial PCr/ATP ratio. In addition, the hearts of DOX-treated animals showed a loss of the intracellular total creatine pool (ΣCr), which indirectly indicated a damage to the sarcolemma [34]. The animals treated with DOX and peptide G retained myocardial ΣAN and ΣCr pools due to maintaining higher levels of ATP and PCr and demonstrated a trend to an increased PCr/ATP ratio. This increase in cardiac energy supply was in good agreement with the higher RCI value in permeabilized myocardial fibers, which reflected better coupling of respiration and phosphorylation in mitochondria. Thus, peptide G attenuated DOX-induced cardiac dysfunction in rats by suppressing oxidative stress and increasing the functional activity of mitochondria.

A chimeric peptide G was synthesized by conjugation of galanin (2–13) with the dipeptide carnosine. When designing this peptide ligand, the following considerations have been taken into account: (i) the first 15 amino acid residues of N-terminal galanin fragments are of critical importance for their biological activity and cardiovascular regulation [35]; (ii) the deletion of the Gly1 residue results in loss of affinity of N-terminal galanin fragments for the GalR1 receptor [36]; (iii) galanin fragments (2–11) and (2–15) having preferential affinity for the GalR2 receptor are able to reduce I/R injury due to antioxidant properties and inhibition of cell death [21, 23]; (iv) the Trp2 and Tyr9 residues, together with Asn5, Gly8, Leu10, and Gly12, are the critical pharmacophores for selective binding of galanin fragments to the GalR2 receptor [37]; (v) a novel agonist should be readily soluble in water to test its activity in animal models *in vivo*. The synthesized ligand G, [βAla14, His15]-galanin (2–15), has a greater solubility in water than the natural galanin fragments (2–11) and (2–15) [22] and enhanced resistance to the action of exopeptidases due to carnosine tailing to C-terminal part of the molecule.

The potency of G as the agonist for galanin receptor subtypes GalR1-3 has not been studied yet, but its preferential affinity to the galanin receptor GalR2 is quite probable. GalR2 receptor signals through several classes of G-proteins and stimulates multiple intracellular pathways that may play a role in attenuation of DOX-induced cardiotoxicity. The most significant of them enhance cell survival by suppressing caspase-3 and caspase-9 activity, mediate the release of Ca^{2+} into the cytoplasm from intracellular stores and opening Ca^{2+}-dependent channels, and cause GLUT4 translocation from intracellular membrane compartments to plasma membranes to enhance glucose uptake [38, 39]. It is possible that peptide G, like the natural galanin fragments (2–11) and (2–15), has antioxidant properties. Since DOX has been shown to reduce the activity of catalase, glutathione peroxidase, and superoxide dismutase [9, 14, 40, 41], a direct

scavenging of ROS or replenishing depleted antioxidant system might be effective in the prevention of DOX cardiotoxicity. Noteworthy, administration of peptide G to healthy animals was not accompanied by potentially detrimental effects or changes in metabolic and functional parameters of the heart. The results indicate that this peptide exerted beneficial effect under oxidative stress and myocardial metabolic disturbances caused by DOX. The exact molecular mechanisms of peptide G-related cardioprotection against DOX-induced damage have yet to be investigated. If such a protective function is confirmed in cancer patients, this novel agonist of galanin receptors can be used in therapy with DOX.

To date, dexrazoxane (DRZ) is the only drug approved for prevention of anthracycline-induced heart failure. Cardioprotective effects of DRZ are attributed to iron chelating by ADR-925, an EDTA-like product of the enzymatic DRZ hydrolysis, and to preventing DOX-induced DNA damage through depleting TOP2B [12, 13]. DRZ is able to reduce damage to sarcolemma and protect the cardiac mitochondria from ultrastructural and functional damage, which are initiated by ROS formation [42]. Pretreatment with DRZ prevents from DOX-induced cardiotoxicity due to inhibition of short-lived ROS formation via TOP2B degradation [11] and an increase in the antioxidant status of the myocardium [43]. However, some works demonstrate that DRZ administration does not attenuate DOX-induced cardiac damage and oxidative stress [44]. We believe that peptide G can be considered as a potential adjuvant to DRZ in preventing cardiotoxicity caused by DOX.

Study Limitations

This study demonstrated beneficial effects of peptide G on rat heart damaged by DOX. However, the effectiveness of peptide G as a protective agent in DOX-induced cardiotoxicity requires additional experimental evidence. This applies primarily to the use of specific cardiac markers such as troponins and quantitative histopathological examination of the heart to verify metabolic and functional effects of the peptide. The role of peptide G in suppressing cardiac oxidative stress should be assessed not only by reducing plasma TBARS concentration, but also by the activity of myocardial antioxidant enzymes and the formation of ROS. These issues will be the subject of further research, in which we intend to study the molecular mechanisms of action of this peptide. In addition, the limitation of the present study is that biochemical and echocardiographic examinations were performed on the day following cessation of treatment with peptide G. This prevented us from determining the long-term cardioprotective effect of this compound. Although peptide G reduced myocardial damage induced by DOX, our model was not designed to assess how this compound affects antitumor properties of DOX.

In conclusion, the current study suggests that peptide G, [βAla14, His15]-galanine (2–15), a novel agonist of galanin receptors GalR1-3, has a protective effect against DOX cardiotoxicity. Co-administration of peptide G with DOX was associated with less damage to cardiac function. This protective effect was caused by the inhibition of oxidative stress and improvement in myocardial energy state. Thus, peptide G can be a promising candidate for protection from DOX cardiotoxicity in patients with cancer.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval All applicable international and national guidelines for the care and use of animals were followed. The experiments with animals were approved by the Bioethical Committee for Animal Care of National Medical Research Center for Cardiology (Permission No. 12 of 19 September 2017).

Informed Consent Informed consent was obtained from all individual participants included in the study.

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