Short Homologous Peptides Based on C-Terminal Sequences of Fibrinogen β - and γ -Chains (Haptides) Affect Cardiovascular Function by eNOS Inhibition

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Key Words

Fibrinogen • Haptides • Nitric oxide • Fibrinolysis • Hemodynamic • Isolated heart, rat • Endothelial nitric oxide synthase

Abstract

Haptides are a family of 19–21-mer cell-binding and permeating peptides homologous to sequences in the C termini on both fibrinogen β - and γ -chain (C β and preC γ , respectively). The effect of the Haptides on the cardiovascular system was studied by different assays, including the activity of isolated perfused rat heart and blood vessels in the organ bath. Haptides (50–80 μ g/ml) decreased the hemodynamic functions of perfused rat hearts by up to 60% ($p < 0.05$) in a dose-dependent manner. Whole fibrinogen or a control nonrelated peptide (C α) did not show such an effect. The NO donor, sodium nitroprusside, reversed the inhibitory effects of Haptides. L-NAME, an endothelial nitric oxide synthase (eNOS) inhibitor, did not further augment the effect of the Haptides. Perfused ¹²⁵I-Haptides were attached to the coronary endothelium. In myocardial homogenates and HUVEC, Haptides significantly decreased eNOS activity, but had no effect on the contraction of isolated cultured adult cardiomyocytes.

Haptides also significantly enhanced the contraction of rings of rat aorta and human mammary artery vessels ex vivo only when the endothelium was intact. Haptides seem to affect the coronary endothelium, but not the cardiomyocytes, by inhibiting eNOS activity, causing vasoconstriction, temporary ischemia and impaired myocardial function that seem to be related to the amino acid composition of the Haptides.

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Introduction

The cardiovascular system is highly affected by fibrin homeostasis [1]. Coagulated fibrin is a main contributor to different vascular occlusive diseases. Induced fibrinolysis is a primary treatment to resolve and prevent such complications. During different cardiovascular procedures, such as stent implantation or cardiopulmonary bypass surgery, the homeostasis is disrupted. Prolonged blood contact with artificial surfaces during the cardiopulmonary bypass and hypothermia may lead to excessive activation of fibrin coagulation. This is prevented by heparin accompanied with a high rate of fibrinolysis [1]. Full comprehension of the multifactorial activation of co-

agulation, fibrinolysis and inflammation mechanisms during cardiopulmonary bypasses and stent implantation may enhance the effectiveness of the procedures and improve the pharmaceutical options.

Fibrinogen, a protein of 340 kDa (Fib 340), is present in the circulation at a concentration of ~2–4 g/l. Upon injury a release of thrombin results in a proteolytic cleavage of fibrinogen that induces blood clotting. Coronary artery occlusion by atheroma and fibrin clot may result in severe myocardial ischemia and infarction [2]. In addition, higher levels of circulating fibrinogen may increase the risk of cardiovascular disorders [2, 3]. Moreover, both levels of fibrinogen and its degradation products seem to be associated with an elevated risk of arterial thrombosis and mortality from coronary heart disease [3].

Fibrinolysis and reperfusion are the most prominent means of salvaging the ischemic heart. The enzyme plasmin induces fibrinolysis by cleavage of the fibrin at specific locations, yielding identifiable soluble fragments [4]. Upon the early phase of fibrinolysis large D-dimers are released [5]. Elevated circulating D-dimers are found in numerous pathological conditions [6–8], and are used for diagnosing vascular thromboembolism [9]. It has been suggested that the D-dimers have proinflammatory effects [10]. D-dimers are further degraded and removed from the circulation as smaller polypeptides [4, 5].

Haptides, a family of short homologous (~20 mer) cell-binding peptides, are equivalent to sequences of the highly preserved C termini of the fibrinogen β -chain (C β), a sequence preceding the C terminus of the γ -chain (preC γ) and a sequence on the extended α -chain α E (C α E), which is missing in adult mammals due to post-translational modification [11]. Preserved homologues of Haptides sequences also appear in other proteins that contain the fibrin-related domain on their C termini, such as microfibril-associated protein 4 and members of the families of the proteins angiopoietin and tenascin (unpubl. data). These peptides were termed 'Haptides' because of their haptotactic (cell-binding) properties [12, 13]. Haptides were initially studied as cell-binding domains on fibrin(ogen), which are not necessarily mediated by integrins [14, 15]. The Haptides are relatively rich in both basic amino acids as well as cyclic hydrophobic groups, and are rapidly internalized into cells and can promote liposome uptake at nonsaturable kinetics, with no apparent cytotoxic effect [12, 13].

Each D-dimer contains 4 Haptidic sequences. Therefore, complete fibrinolysis produces smaller fragments of the C termini of fibrin which should contain significant

levels of the Haptidic sequences [16, 17]. The present study examines the interaction of the Haptides with the cardiovascular system, based on our hypothesis that Haptides alone, or as part of fibrin degradation products (FDP), may affect heart function. Our findings indicate that Haptides, as well as whole FDP that are released upon fibrinolysis, exhibit hemodynamic effects in the isolated perfused rat heart, probably through endothelial nitric oxide synthase (eNOS) inhibition.

Materials and Methods

Chemicals and Reagents

Human fibrinogen and thrombin were obtained from Omrix tissue sealant (Israel). Sodium nitroprusside (SNP), N ω -nitro-L-arginine methyl ester (L-NAME), collagenase A, and human plasmin were supplied by Sigma (Israel). Tris-HCl gel, Tris/glycine, protein molecular weight standards and Coomassie staining reagents were supplied by Bio-Rad (Israel). β -Mercaptoethanol was supplied by Promega (Israel).

Krebs-Henseleit (KH) solution was prepared daily according to Neely and Rovetto [18], containing 118 mM NaCl, 4.9 mM KCl, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, 11.1 mM glucose, and 2.5 mM CaCl₂. The KH solution was aerated with a mixture of 95% (v/v) oxygen and 5% (v/v) carbon dioxide.

Synthesis of Haptides

The synthetic Haptides C β (KGSWYSMRKMSMKIRPFF-PQQ) and preC γ (KTRWYSMKKTTMKIIPFNRL) correspond to addresses 441–461 of the C terminus of fibrinogen β -chain and 373–392 of the γ -chain, respectively. Cmfa (KGFYYSLKRTEM-KIRRA) corresponds to the address 239–256 on the C terminus of microfibril-associated protein-4. The sequence of arbitrarily scrambled sequences of C β (scr-C β) and preC γ (scr-preC γ) were ISSWYKFPMQGGQKRPRMSFKM and FIKLMNPWSTRYIK-MRTKTK, respectively. The control peptide C α (SEADHEGTH-STKRGHAKSRP) corresponds to a sequence close to the C terminus of the fibrinogen α -chain. The peptides were synthesized by GeneScript (Piscataway, N.J., USA). The experiments employed peptides that were >90% pure as determined by HPLC and mass spectrometry. Fluorescein isothiocyanate (FITC)-labeled peptides were also synthesized with a single FITC tag at the amino terminal.

Animals

Male Sprague-Dawley rats (275–300 g; Harlan Laboratories, Israel) were grown in specific pathogen-free conditions. All animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals (www.nap.edu/catalog/5140.html). The experiments were approved by the Institutional Animal Care and Utilization Committee.

Isolated Perfused Rat Heart Model

Rats were anticoagulated with sodium heparin (i.p., 500 U/rat) and 30 min later anesthetized with sodium pentobarbital (i.p., 30 mg/rat). Hearts were immediately removed and placed in hepa-

rinized ice-cold saline solution. The aorta was cannulated to a Langendorff perfusion system, as previously described [19]. Hemodynamic parameters were monitored using a latex balloon-tipped catheter inserted into the left ventricle as previously described [19]. Left-ventricular systolic and diastolic pressures and time derivatives of pressure (dp/dt_{max}) were measured during contraction and relaxation. Left ventricular developed pressure (LVDP) was calculated as the difference between the systolic and diastolic pressures. The work index was derived from the product of heart rate (HR) and LVDP ($LVDP \times HR$). Coronary flow rate (CF) was measured by collecting the effluent drained through the pulmonary artery during perfusion.

Coronary vascular resistance ($mm\ Hg \cdot min \cdot g \cdot ml^{-1}$) was calculating as follows:

$$CVR = (P_{per}/CF) \times DW$$

where CVR is coronary vascular resistance, and P_{per} is the perfusion pressure (70 mm Hg), which is kept constant. DW is the dry weight of the heart.

Following 20 min of equilibration perfusion, peptides dissolved in KH were infused into the heart through a side arm for 2 min, and the hemodynamic parameters were measured for an additional 10 min. Following 10 min of KH perfusion rinse, the heart was exposed to KH or a higher dose of the peptide dissolved in KH (for an additional 2 min), and the hemodynamic parameters were measured for another 10 min. The maximal effect of the Haptides was recorded after 2 min. The side arm infusion into the heart was performed by a syringe pump adjusted to take into account heart CF rate, and was equal to 1/20 of CF for each heart. Thus, the final concentration of the peptide or FDP reaching the isolated heart was 1/20 of that in the syringe.

Frozen Sections for Fluorescence Microscopy

Following $FITC$ -Haptides perfusion (80 $\mu g/ml$) for 2 min, the isolated hearts were frozen in liquid nitrogen. Cryosections (6 μm thick) were mounted on poly-L-lysine-coated slides and were stained with hematoxylin. Light and fluorescent microscopy for $FITC$ (488 nm) was performed in parallel.

Effect of Haptides on Amplitude of Shortening in Isolated Adult Rat Cardiomyocytes

Isolated adult rat cardiomyocytes were prepared enzymatically as previously described [20]. The cells were suspended in KH containing 10% albumin and were plated on culture plates. Two platinum electrodes (1 mm apart) connected to a stimulator (Grass Instruments, Quincy, Mass., USA) created 2-ms square-wave bipolar pulses at 0.5 Hz, which stimulated the KH-perfused myocytes. The cell images, collected by ultraviolet epifluorescence objective ($\times 40$), were diverted to the microscope's side port, and recorded by a video camera (Pulnix, Sunnyvale, Calif., USA). Amplitude and velocity of shortening of the myocytes were analyzed by a video edge.

eNOS Activity in Myocardial Homogenate

The fluorometric assay of eNOS activity in heart homogenates was performed according to the previously described assay [21]. Essentially, 20% (w/v) rat heart homogenate was prepared in Tris-HCl buffer, pH 7.5, with 1 mM EDTA, 1 mM dithiothreitol, 10 $\mu g/ml$ antipain, leupeptin, and pepstatin A and 0.1 mM phenylmethylsulfonyl fluoride. For the standard eNOS assay, a reaction mixture

was prepared based on 100 mM Pipes-NaOH buffer, pH 7.0 with 100 μM arginine, 15 mM NADPH, 1.25 mM $CaCl_2$, 1 μg calmodulin/ml, 1 mM dithiothreitol, 1 mM EDTA, 3.0 mM nicotinamide, 10 $\mu g/ml$ antipain, leupeptin, and pepstatin A and 0.1 mM phenylmethylsulfonyl fluoride. Reaction mixture B contained 500 mM L-NAME in addition to all components in reaction mixture A. The difference in the activities between reactions A and B represented eNOS-specific activity. The activity assayed in the reaction mixture B is nonspecific to eNOS $NADP^+$ formation. Reaction mixture (100 μl) A or B was preincubated at 25°C for 5 min and 25 μl of the heart homogenate was added for 5 min. The reaction was stopped by 10 μl of 1 M HCl containing 10 mg/ml pepsin. Following incubation at 38°C for 30 min, the HCl activates the pepsin which destroys the enzymes (eNOS) and nonreacted NADPH. The assay was developed for 1 h at room temperature in the dark by the addition of 1 ml of 6.6 M NaOH with 2 mM imidazole at room temperature. The reading was made by fluorometer (Ex 370, Em 465; Crescent Electronics, Salt Lake City, Utah, USA) and the computer data acquisition system.

Activity of Rat Aortic Rings in an Organ Bath

Sprague-Dawley male rats (250–280 g) were injected intraperitoneally with sodium heparin and after 30 min were anesthetized with pentobarbital sodium (i.p., 30 mg/rat). The rats were killed by decapitation. The thorax was opened and the thoracic aorta (aortic arch to the diaphragm) was rapidly excised and transferred into cold KH. A portion of the extracted aorta was trimmed of surrounding connective and fat tissues and cut into ~3-mm-wide rings (~3 mg). The aortic rings were suspended between two stainless steel hooks immersed in KH (37°C, aerated with 95% O_2 /5% CO_2) inside the organ bath chamber. The baseline tension of the rings was adjusted to 2 g that was found to be optimal [22], and equilibrated for 60 min.

Maximal contraction of the rings was induced by 80 mM KCl. Then, the chambers were rinsed by 3 exchanges of the KH for 45 min, while the tension was adjusted to baseline value (2 g). Intact aortic rings were exposed to 40 $\mu g/ml$ of the tested peptide and the contraction was recorded for 10 min. Next, 60 $\mu g/ml$ of the tested peptide was added, and the contraction was recorded again for 10 min.

The endothelium was scraped by mechanically cutting it off by insertion and rolling of a polyethylene tube (PE50) and gauze into the lumen of the aortic rings. The rings were then exposed for 10 min to 40 mM KCl, in order to achieve 50–60% of maximal contraction. Acetylcholine (10^{-5} M) was added. Following the rinsing of the chambers for 45 min by 3 exchanges of the KH, the tension was readjusted to baseline value (2 g). Only the rings that contracted in response to 40 mM KCl but did not relax in response to acetylcholine (indicating successful de-endothelialization) were further examined with the peptides. The rings were exposed to 60 $\mu g/ml$ of the tested peptide and the contraction was recorded for 10 min.

Human Internal Mammary Artery Activity in Organ Bath

Viable segments of internal mammary artery were obtained from discarded tissue of surgery on patients undergoing first-time, nonemergent coronary artery bypass graft operation without concomitant valve surgery. The use of these materials of human origin was approved by the Institutional Helsinki Committee and written informed consent was obtained from all involved patients.

Mammary artery activity in the organ bath was performed as previously described [23]. Essentially, segments of the freshly dissected conduits were immediately placed in ice-cold KH. The excised vessels were divided into 3- to 4-mm segments that were suspended between two stainless steel stirrups in 10-ml organ chamber baths and constantly aerated with 5% CO₂ 95% O₂. After a 20-min adaptation period, each vessel segment was stretched to approximately 20% of optimal resting tension, to produce a maximal contractile response to 2.0 M KCl. Vessels were then washed 3 times to completely remove the excess KCl. Thirty minutes later, they were exposed to 40 µg/ml of one of the tested Haptides or the control peptides Cα.

Assay of NO Secretion by Endothelial Cells

Human umbilical vein endothelial cells (HUVEC) were obtained and cultured as previously described [24]. Experiments were conducted on confluent cells between passages 2 and 3. Endogenous NO synthesis was assessed following incubation of the cells with either 50 µg/ml of the different peptides or with the 20 ng/ml of VEGF (positive control) for 2, 15 or 120 min. At the different time points, 50 µl of the supernatant was taken for the determination of nitrite/nitrate production. The concentration of inorganic nitrites (NO₂) and nitrates (NO₃), the stable end products of NO, was assayed by a colorimetric method using a Kit that determines NO₂/NO₃ based on the Griess reaction (Cayman Chemical Co., Ann Arbor, Mich., USA) [25]. The NO₂/NO₃ reading was then normalized to the number of endothelial cells per well (10⁵ cells). The results are presented as percent of the NO level of the nontreated cells for the same time points.

Preparation of FDP

We used clinical grade human fibrinogen (>95% coagulable) and human thrombin from fibrin glue kits (Vitex Inc., New York, N.Y., USA). Fibrinogen was dialyzed against carbonate buffer (pH 8.2). Protein concentration was determined by the Bradford dye method, using pure fibrinogen as a calibrant. Fibrinogen and thrombin were dissolved and diluted in a buffer of 10 mM Tris/saline (pH 7.4). To induce polymerization of fibrinogen monomers, 1 ml of thrombin (0.08 U/ml) with 1 mM CaCl₂ from the same source was added to 2 ml of fibrinogen. Clot was induced in 37°C and was allowed to stabilize for 1 h. Then the clot was rinsed extensively with Tris buffer. FDP were obtained by incubation of the fibrin in a solution of human plasmin (~0.08 U/ml) in 37°C with 1 mM CaCl₂ for 4 h. FDP were taken for isolated heart assay.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

FDP (0.6 mg/ml) were analyzed on 12% polyacrylamide gel electrophoresis at reducing conditions (2-mercaptoethanol and boiled for ~5 min).

Statistical Analysis

All values are expressed as mean ± SE. One-way ANOVA and a 2-tailed Student t test were used for the comparison between relevant groups. Statistical differences of $p < 0.05$ were considered significant.

Results

Effect of Haptides on the Isolated Perfused Heart Function

Preliminary experiments indicated that the concentrations of Haptides that profoundly affect myocardial function are higher than 50 µg/ml (data not shown). The concentrations that were tested extensively in the isolated perfused heart model were in the range of 50–80 µg/ml. Two minutes of exposure of the hearts to 50 µg/ml of Haptides, either Cβ, preCγ or Cmfa, significantly decreased the dP/dt_{max} , HR, heart work index (LVDP × HR) and CF ($p < 0.05$). A KH perfusion rinse for 10 min resulted in almost complete recovery of myocardial function (fig. 1a–d, respectively).

Following the 10 min of KH perfusion rinse, the same hearts were exposed to a higher concentration (80 µg/ml) of the Haptides Cβ, preCγ and Cmfa. In general, the higher concentration further decreased all hemodynamic parameters very significantly (fig. 1a–d). The following 10-min KH perfusion rinse resulted only in a partial hemodynamic recovery (fig. 1a–d). The control peptide Cα or the fibrinogen protein (50 and 80 µg/ml) did not affect the hemodynamic parameters (fig. 1a–d). Of interest is that scramble sequences of both Cβ and preCγ that were prepared as arbitrary rearrangement of the peptide sequence also produced similar effect as the parent Haptides in all the parameters studies. For example, the effect of the same concentration of scrambled sequences on the work index of the heart is presented in figure 1e.

Effect of Haptides on the Coronary Artery Resistance

Haptides Cβ, preCγ and Cmfa (50 µg/ml) significantly elevated the coronary resistance ($p < 0.05$), while the KH perfusion rinse relieved it (fig. 2a). A higher concentration (80 µg/ml) of the Haptides Cβ, preCγ and Cmfa further elevated the coronary resistance ($p < 0.005$). The KH perfusion rinse partially reversed the coronary resistance elevated by Haptides. In contrast, the same concentrations of control peptide Cα or fibrinogen had no effect (fig. 2a).

When the effect of Haptides was tested on aortic rings in the organ bath, a dose-dependent contraction effect was seen (fig. 2b). Both Haptides Cβ and preCγ (60 µg/ml) induced similar significant contractions of the intact aortic rings ($p < 0.005$, fig. 2c). The mediated contraction of the Haptides was not apparent in aortic rings with scraped endothelium (fig. 2d). Furthermore, in intact human mammary artery rings Haptides Cβ and preCγ (40 µg/ml) induced similar significant contraction ($p <$

Fig. 1. Haptides decrease myocardial function. Isolated perfused rat hearts were exposed to 2 doses (50 and 80 μ g/ml) of the Haptides: C β , preC γ , Cmfa, control peptide C α or whole fibrinogen (n = 6). The results (mean $\pm$ SE) are presented as % of the baseline values: dP/dt_{max} (a), HR (b), work index = LVDP $\times$ HR (c), and CF (d). The effects are compared to the control C α peptide. **e** Effect of scrambled Haptides sequences scr-C β and scr-preC γ on heart work index. * p < 0.05, ** p < 0.01.

Fig. 2. Haptides elevate the coronary resistance. **a** Isolated perfused rat hearts were exposed to 2 doses (50 and 83 $\mu\text{g/ml}$) of Cmfa, C β , preC γ , C α or whole fibrinogen for 2 min, separated by 10 min of KH perfusion rinse. The resistance of the coronaries was assessed as described in Materials and Methods. The results (mean $\pm$ SE, $n = 6$) are presented as % of the baseline values (* $p < 0.05$, ** $p < 0.01$ compared to the control C α peptide). **b** Dose-dependent effect of C β , preC γ and C α peptides (40 and 60 $\mu\text{g/ml}$) on the contraction of intact aortic rings; tracing of a

representative experiment. Effect of Haptides C β , preC γ or the control C α peptide (60 $\mu\text{g/ml}$) on aortic ring contraction in intact rings (**c**) or rings with scraped endothelium (**d**). The contraction effect of the Haptides (C β , preC γ) is not apparent in rings with scraped endothelium (**d**). **e** Effect of Haptides C β , preC γ or the control peptide C α (40 $\mu\text{g/ml}$) on the contraction of intact human mammary artery rings. Results (mean $\pm$ SE, $n = 6$) are expressed as % of the maximal contraction (in the presence of 80 mM KCl; ** $p < 0.01$).

0.005, fig. 2e). This effect seems to be not related to prostaglandins since their inhibitors such as indomethacin did not affect Haptide-induced aortic ring contraction (data not shown).

Effect of FDP on Isolated Perfused Heart Function

SDS-PAGE of fibrinogen resulted in 55- to 65-kDa fragments corresponding to the separate fibrinogen chains (α , γ and β), in addition to a 340-kDa band corresponding to the complete fibrinogen molecule (fig. 3a). Plasmin cleavage of fibrin resulted in FDP. Many of the fragments of the D-dimers of the size of less than below

100 kDa contain the full sequences of the Haptide sequences in their C termini (fig. 3a).

FDP was less effective than the Haptides in decreasing the hemodynamic parameters in the perfused heart; 150 $\mu\text{g/ml}$ of FDP did not affect the hemodynamic functions (fig. 3b–e). A second pulse of a higher dose of FDP (250 $\mu\text{g/ml}$) significantly decreased dP/dt_{max} , work index and the CF ($p < 0.05$). Perfusion rinse with KH resulted in a full recovery of the hemodynamic parameters. Pulses of 1.5 and 2.5 mg/ml of whole fibrinogen or 2×10^{-3} U/ml and 6×10^{-3} U of plasmin did not affect the myocardial function (fig. 3b–e).

Fig. 3. Effect of FDP on myocardial function. Isolated perfused rat hearts were exposed to 2 doses of FDP (150 and 250 $\mu\text{g/ml}$), to plasmin (2×10^{-3} and 6×10^{-3} U/ml) or fibrinogen (1.5 and 2.5 mg/ml) ($n = 4$). **a** SDS-PAGE of FDP and whole fibrinogen. 1 = Molecular weight standards; 2 = fibrinogen (10 μg); 3 = fibrinogen (10 μg) after incubation with plasmin; 4 = fibrin (10 μg) after incubation with plasmin; 5 = fibrinogen (30 μg) after incubation with plasmin; 6 = fibrin (30 μg) after degradation by plasmin. **b** dP/dt_{max} . **c** HR. **d** Work index. **e** CF. The results (mean $\pm$ SE)

are presented as % of the baseline value. The arrows indicate the time points of exposure to the different agents. The left arrow is for the low dose of the agents tested (150 $\mu\text{g/ml}$ of FDP, 2×10^{-3} U/ml of plasmin enzyme and 1.5 mg/ml of fibrinogen). The right arrow is for the high dose of the different agents tested (250 $\mu\text{g/ml}$ of FDP, 6×10^{-3} U/ml of plasmin and 2.5 mg/ml of fibrinogen). The statistical significance was tested in comparison to the control group treated with plasmin (* $p < 0.05$).

Localization of the Perfused ^{FITC}Haptides in the Heart

Frozen sections from hearts perfused with ^{FITC}Haptides revealed attachment of the Haptides to the endothelium of the coronary arteries with some penetration. No attachment of Haptides to the cardiomyocytes was observed (fig. 4). The control peptide ^{FITC}C α did not attach to the coronary arteries or the cardiomyocytes (fig. 4).

Effect of the Haptides on the Contraction of Isolated Cultured Cardiomyocytes

The direct effect of Haptides on isolated adult rat cardiomyocytes was further examined by using video motion assay. Incubation for 2 and 5 min with the Haptides

preC γ and C β , as well as with the control peptide C α (50 and 100 $\mu\text{g/ml}$), did not affect the amplitude of contraction of the cardiomyocytes (fig. 5).

Effect of the Haptides on eNOS Activity

L-NAME, an eNOS inhibitor, and SNP, an NO donor, were studied for their modulation of the Haptide C β effect on isolated perfused hearts. As expected, L-NAME alone decreased the myocardial hemodynamic function, similar to the effect of C β alone. A combined treatment of C β with L-NAME had no additive inhibitory effect. SNP alone had no significant effect on the myocardial function, but it fully reversed the inhibitory effect of C β

Fig. 4. Haptides ^{FITC}Cmfa and ^{FITC}Cβ attachment to myocardial endothelium. Frozen sections were prepared following exposure of the isolated hearts to ^{FITC}Cmfa, ^{FITC}Cβ, ^{FITC}preCγ or to the control peptide ^{FITC}Cα (n = 2). **a** Longitudinal section. **b–d** Transversal myocardial sections. The arrows indicate Haptides binding to the vascular endothelium.

Fig. 5. Haptides preCγ, Cβ, as well as Cα control peptide do not affect contraction of isolated adult cardiomyocytes. Adult cardiomyocytes were isolated and incubated for 5 min with 100 μg/ml Haptides (preCγ, Cβ) or Cα control peptide. Assessment of contraction was performed by video motion as described in Materials and Methods. The results (mean ± SE, n = 3) are presented as % of the baseline initial values.

on the hemodynamic function of the isolated hearts (fig. 6a).

The direct effect of Haptides on eNOS activity was also studied in heart homogenate. While the control Cα peptide did not inhibit eNOS activity, Cβ or preCγ (100 μg/ml) significantly decreased eNOS-mediated NADP⁺ production (fig. 6b). These data indicate that the effect of Haptides is associated with increased coronary resistance, due to direct interaction with the endothelium causing decreased activity of the eNOS.

Incubation of HUVEC with the Haptide Cβ, preCγ or Cmfa resulted in an NO level decrease within 2 min, which persisted for up to 15 min. In a longer period, the effect of Haptides on NO synthesis fades. VEGF that served as positive control gradually increased NO secretion, while Cα that served as a negative control did not seem to have any effect on NO secretion (fig. 6c).

Discussion

The present study demonstrates that Haptides which are fibrin-related peptide sequences that also appear in the C termini of other proteins have major effects on the heart. For instance, fibrin-related Haptides that are re-

Fig. 6. Haptide-mediated inhibition of eNOS activity. **a** The effect of Cβ (50 μg/ml) alone, Cβ (50 μg/ml) with L-NAME (3 mM), L-NAME (3 mM) alone, Cβ (50 μg/ml) with SNP (10⁻⁵ M) and SNP (10⁻⁵ M) alone on the hemodynamic parameters in an isolated perfused heart. The results (mean ± SE, n = 4) presented as % of the baseline hemodynamic parameters (100%, shown by a broken line). Statistical significance was tested in comparison to the Haptide Cβ group (* p < 0.05, ** p < 0.01). **b** The Haptides Cβ and preCγ but not the peptide Cα (100 μg/ml) significantly inhibit eNOS activity in myocardial homogenates. The results (mean ± SE, n = 3) are presented as % of control NADP⁺ production (without the peptide). **c** Determination of the NO level in cultures of HUVEC. Measurements of NO levels were done at 2, 15 and 120 min. While Cα served as a negative control, VEGF that induces NO was used as a positive control. The data are presented as % of the NO level in untreated cells at the same time points. The results represent an average of the data of the experiments performed in duplicate.

leased as a result of pathological cardiovascular events may adversely affect cardiac function. Previous studies revealed that synthetic Haptides dissolved as a monomeric form in aqueous solution penetrated and accumulated in different cell types, including fibroblasts, and endothelial and smooth muscle cells [13]. With the elevation of their concentrations they created nanoaggregates, which interacted with membranes and rapidly penetrated into the cells at nonsaturable kinetics and even enhanced penetration of liposomal compositions into them, acting as

classic cell-penetrating peptides [12, 13]. Of note is that in monolayer cell culture the penetration of Haptides into the cells was not associated with any alteration in their survival or proliferation rate [13].

In the current study, isolated rat heart exposure to Haptides in concentrations of more than 50 μg/ml significantly increased the rat coronary artery resistance and thereby caused a negative inotropic and chronotropic effect (fig. 1a–d). This effect was robust and only partially reversible at a higher concentration of 80 μg/ml.

Fig. 7. Proposed model for the mechanism of Haptide-mediated regulation of eNOS with subsequent effect on the coronaries. Our proposed model is based on a previously described mechanism of NO regulation of blood vessel tonus [28]. It proposes that the Haptidic sequences, either as synthetic peptides or as part of the FDP, inhibit eNOS activity by a cascade of events (marked by red). The Haptides inhibit eNOS and thereby reduce NO synthesis. This inhibits guanyl cyclase activity and reduces cGMP in the system. Subsequently, the K⁺ channels (BKCa) are blocked. The positive charge of the membrane is conserved and prevents closing of the voltage-dependent Ca²⁺ channels in the membrane. The subsequent leakage of Ca²⁺ into the smooth muscles results in vasoconstriction of the coronary arteries.

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Furthermore, to support this finding on the vascular-related adverse effects, we observed that the active Haptides also significantly increased the contraction of rings of rat aorta as well as human mammary arteries (fig. 2). The effect of the Haptides on the isolated perfused heart was immediately apparent, thus excluding a mechanism related to the induction of gene expression. The 3 homologous Haptides tested, including Cmfa (which is not derived from fibrin), had a similar effect. The fact that their scrambled versions are also similarly active indicates that the effect of these homologous peptides is due to the amino acid composition and not the sequence that triggers the recorded cardiovascular effects. In contrast, peptide C α of a different composition that was used as a control did not affect myocardial function.

Each D-dimer contains 4 Haptidic sequences. Therefore, complete fibrinolysis produces smaller fragments of the C termini of fibrin which should contain significant levels of the Haptidic sequences [16, 17]. According to previous reports [4, 5], the immediate degradation products of fibrin by plasmin digestion contain the C termini of the β - and γ -chains as large fragments of less than 100 kDa (the size of a single D residue of the fibrin). Previous studies showed that low-molecular-weight FDP could penetrate the endothelial cell membrane [26]. Due to their large size, their penetration into cell membranes may still be limited and the relative composition of Haptides within these polypeptides is also relatively small.

This may explain the lower, but still significant, effect of FDP relative to the effect of the small synthetic pure Haptidic sequences.

In specific pathological conditions like the treatment of pulmonary embolism, deep venous thrombosis and disseminated intravascular coagulation, there is an increase in the systemic level of such fragments containing Haptides [6–9]. Since these large Haptides containing fragments can penetrate into endothelial cells of blood vessels in large quantities, they may affect the coronary and thereby the heart functions as suggested in figure 3.

Histological examination revealed that the perfusion of fluorescent Haptides (^{FITC}C β or ^{FITC}Cmfa) in the isolated heart system resulted in their accumulation in the intima of the coronary vessels, with no apparent binding to the cardiomyocytes (fig. 3). The effect of Haptides was found to be endothelial dependent, since they induced aortic ring contraction ex vivo, but did not affect the aortic rings in which the endothelium was scraped. Moreover, C β and preC γ did not affect the contraction of isolated beating cardiomyocytes, which suggested that their target may not be the cardiomyocytes (fig. 4).

Vascular function is regulated by various vasoactive mediators such as NO [27, 28]. It has been suggested that acute thrombosis may be a result of endothelial dysfunction due to decreased endothelial-dependent blood vessel relaxation caused by a diminished local NO level [26–31]. In addition, in thrombolysis L-arginine (NO precursor)

caused amelioration of endothelial dysfunction [32]. In relation to our findings, thrombolysis may release high levels of Haptide-containing sequences that may depress NO levels and cause the vasoconstriction that may impair CF.

Haptides decreased myocardial function similarly to the eNOS inhibitor L-NAME. On the other hand, the combination of a Haptide and L-NAME did not augment the inhibitory effect on the heart. Exposure of the heart to both C β and SNP (NO donor) relieved the inhibitory effect of C β (fig. 6a). In addition, the eNOS assay indicated that Haptides inhibited NADP⁺ production in myocardial homogenate (fig. 6b). Moreover, Haptides seemed to inhibit NO synthesis quite immediately in cultured monolayer HUVEC (fig. 6c). Thus, these results suggest that the interaction of Haptides with cell membranes and penetration which confirm previous observations of endothelial cells [13] may be associated with the regulation of endothelial NO synthesis. Haptides are relatively rich in basic amino acid arginine and lysine as well as cyclic and hydrophobic groups. This may be the basis for the mechanism of their cell adhesion and fast cell internalization, not through a specific receptor [12]. The control peptide C α that was arbitrarily chosen does not have a high content of net positively charged and hydrophobic residues and this may explain its lack of activity.

A main indication that the cardiovascular effects of the Haptide were not mediated by a specific receptor was a similar effect which was recorded with the use of scrambled sequences of both fibrin-related Haptides (fig. 1e). This conforms with the previously described in vitro inhibitory effects of positively charged hydrophobic peptides on eNOS activity, which may compete with calmodulin binding and thereby reduce NO synthesis [33, 34].

Besides its effect on the vascular system, NO may directly affect myocardial contractility, depending on its origin [35]. In the absence of exogenous NO, the contractility is decreased by NOS inhibitors (both in vitro and in

vivo), suggesting that basal endogenous NO production supports the myocardial contractility [36]. Our observations indicate that Haptides that penetrate the endothelial cells may directly inhibit the eNOS activity, and thereby endogenous NO production, causing a decrease in myocardial contractility and hemodynamic function as schematically presented in figure 7.

Our findings suggest that peptidic fragments of FDP containing Haptidic sequences may adversely affect the cardiovascular system. At different pathological conditions, stent implantation and cardiopulmonary bypass surgery, the concentration of Haptide-containing sequences may dramatically increase in the circulation, due to clotting and fibrinolysis processes. Thus, we anticipate that such Haptide-containing fragments released into the circulation may adversely affect the cardiovascular system at a wide range of cardiovascular pathological conditions by their direct effect on the vascular endothelium. In vivo studies are being performed in order to assess the effect of circulating Haptides on the whole animal.

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