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Effects of arginine substitutions on the cardioinhibitory activity of the Led-NPF-I neuropeptide

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Abstract: Effects of structural changes on the cardioinhibitory activity of the Led-NPF-I peptide (Ala-Arg-Gly-Pro-Gln-Leu-Arg-Leu-Arg-Phe-amide) were examined by replacing Arg residues in positions 2, 7 and 9. Replacement of L-Arg² with another basic amino acid, such as Lys, His or D-Arg, did not abolish but rather promoted cardioinhibitory activity in giant mealworm beetle *Zophobas atratus* Fab. Agonistic peptides were also obtained by substitution of Arg residue in position 7 with Lys or D-Arg, and Arg in position 9 with His or D-Arg, respectively. All these analogues showed stronger cardioinhibitory effects than the native peptide at low concentration (10^{-9} M), and [Lys⁷]-, [D-Arg⁷]- and [D-Arg⁹]-Led-NPF-I also at the higher concentration (10^{-6} M). However, substitutions of the Arg residues in position 7 with His or in position 9 with Lys caused a loss of the cardioinhibitory activity. In addition, the replacement of Arg residues in all three positions with Lys or Orn caused a reduction of cardioinhibitory activity, although a single substitution of Arg in positions 2 or 7 with Lys yielded agonistic peptides. We conclude that the Arg² position in the *N*-terminal region is more tolerant to structural modification than the other two Arg positions located in the *C*-terminal region.

Keywords: Led-NPF-I peptide, cardioinhibitory activity, structure-activity studies, *Zophobas atratus* beetle

INTRODUCTION

In insects, the first short peptide hormones related to the vertebrate neuropeptide F (sNPFs), were identified in the head extract of the mosquito *Aedes aegypti* (Aea-NPF-I, Aea-NPF-II) [1] and in the brain of the Colorado potato beetle, *Leptinotarsa decemlineata* Say (Led-NPF-I, Led-NPF-II) [2]. Additional bioanalogues were found in *Schistocerca gregaria* Forsk. [3], *Drosophila melanogaster* Meig. [4], *Helicoverpa zea* Bod. [5], *Periplaneta americana* L. [6], *Anopheles gambiae* Giles [7], and *Tribolium castaneum* Herbs. [8]. All sNPFs identified to date have a typical C-terminal R/K-X₁-R-X₂ amide motif, where the first amino acid is basic (Arg or Lys), X₁ can be Leu, Thr or Pro, and X₂ is always an aromatic amino acid residue such as Phe or Trp.

Insect sNPFs may function within the nervous system as neurohormonal regulators and in physiological processes related to feeding and reproduction. For example, injection of the heterologous Led-NPF-I stimulates ovarian development [9] and increases food intake in *Locusta migratoria* L. [10]. Manipulation of NPFs genes affects food consumption in both larvae and adults of *D. melanogaster* [11].

In vitro studies revealed a cardioinhibitory action of two *L. decemlineata* peptides, Led-NPF-I and Led-NPF-II in *Tenebrio molitor* L. and *Zophobas atratus* Fab. [12, 13]. In preliminary structure-activity studies we showed that the C-terminal pentapeptide fragment Leu-Arg-Leu-Arg-Phe-amide of Led-NPF-I, which is also present in the second *L. decemlineata* peptide Led-NPF-II, is essential for the cardioinhibitory action [13]. Removal of the amide or replacement of the C-terminal hydrophobic Phe with a more polar Tyr or D-Phe isomer caused a loss of activity *in vitro* [14]. It was also observed that in the case of C-terminal amidation Arg²⁻, Pro⁴⁻, Arg⁷⁻ and Arg⁹⁻ residues are required to preserve the peptide inhibitory action on the heart. Moreover, it was found that replacement of Arg residues at all three positions 2, 7 and 9 with similarly basic His residues, reduced cardioinhibitory action of Led-NPF-I, suggesting a functional significance of Arg residues for the peptide interaction with its receptor(s) in the heart [14]. These facts prompted us to further evaluate pharmacological specificity of the response elicited by Led-NPF-I in insect heart.

In the present paper we compared cardioinhibitory action of Led-NPF-I with a series of its analogues on the *Z. atratus* heart. We analyzed the roles of three basic residues of Arg at positions 2, 7 and 9 for the preservation of cardioinhibitory properties of the Led-NPF-I molecule.

MATERIALS AND METHODS

Insects

Zophobas atratus Fab. adults were obtained from a culture maintained at the Department of Animal Physiology and Development as described previously [15].

Peptides

Led-NPF-I (I) and its analogues were synthesized by the classical solid-phase method according to the Boc procedure. As a coupling reagent dicyclohexylcarbodiimide (DCC) or HBTU, both in the presence of HOBt, were used. The Boc protecting group was removed with 30% CF_3COOH in CH_2Cl_2 . Peptides were released from the resin using $\text{CF}_3\text{SO}_3\text{H}$. All peptides were purified on a Sephadex G-15 column and finally by preparative HPLC on C-18 column (Tosoh Bioscience). The following analogues were synthesized for studies: [Lys²]- (II), [His²]- (III), [D-Arg²]- (IV), [Lys⁷]- (V), [His⁷]- (VI), [D-Arg⁷]- (VII), [Lys⁹]- (VIII), [His⁹]- (IX), [D-Arg⁹]- (X), [Lys², Lys⁷, Lys⁹]- (XI) and [Orn², Orn⁷, Orn⁹]-Led-NPF-I (XII). Sequences and physicochemical data of all the peptides are presented in Table 1.

Heart bioassay

Peptides were assayed *in vitro* in a semi-isolated heart prepared according to Rosiński and Gade [16]. In the bioassay, a video microscopy technique and computer-based method of data acquisition and analysis were used to study the action of the peptides on continuously perfused heart preparations. The details of the bioassay have been described elsewhere [14]. Some examples of the records of the heart contractile activity are presented in Figure 1. The activities of tested peptides are expressed as percentage changes in the control frequency of the heart contraction.

RESULTS AND DISCUSSION

The heart contraction frequency of *Z. atratus* remained regular during perfusion with physiological saline for 4-5 h and showed on average 30 ± 4 beats per min. The application of Led-NPF-I caused a fast and reversible decrease of the heart contractile activity (a negative chronotropic effect) (Figure 1).

Table 1. Sequences and physicochemical data of the peptides modified in position 2, 7 and 9 of the peptide chain

	Peptide	Yield [%]	$[\alpha]_D^{20}$ c=0.97% CH ₃ OH	HPLC Rt ^a	Aminoacid analysis	MW Calc. Found	TLC ^b Rf X Y W
I	H-Ala-Arg-Gly-Pro-Gln-Leu-Arg-Leu-Arg-Phe-NH ₂	74.4	-21.7	28.6	Ala 1.0 Arg 3.1 Gly 1.04 Pro 1.1 Gln 0.97 Leu 2.1 Phe 0.97	1212.4 1212.2	0.11 0.51 0.06
II	H-Ala-Lys-Gly-Pro-Gln-Leu-Arg-Leu-Arg-Phe-NH ₂	56	23.8	25.2	Ala 1.05 Lys 0.99 Gly 1.04 Pro 1.1 Gln 1.0 Leu 2.0 Arg 2.1 Phe 0.99	1184.4 1183.8	0.24 0.33 0.18
III	H-Ala-His-Gly-Pro-Gln-Leu-Arg-Leu-Arg-Phe-NH ₂	68	-21.5	26.6	Ala 0.99 His 1.0 Gly 1.04 Pro 1.1 Gln 1.0 Leu 2.1 Arg 2.1 Phe 0.97	1193.4 1192.8	0.20 0.41 0.11
IV	H-Ala-D-Arg-Gly-Pro-Gln-Leu-Arg-Leu-Arg-Phe-NH ₂	59	-35.7	25.5	Ala 1.0 Arg 3.1 Gly 1.0 Pro 0.99 Gln 0.97 Leu 2.1 Phe 1.0	1212.4 1211.7	0.34 0.50 0.17
V	H-Ala-Arg-Gly-Pro-Gln-Leu-Lys-Leu-Arg-Phe-NH ₂	55	-19.8	25.2	Ala 1.0 Arg 2.1 Gly 1.0 Pro 1.0 Gln 1.0 Leu 2.0 Arg 2.1 Phe 0.9	1184.4 1184.7	0.22 0.43 0.18
VI	H-Ala-Arg-Gly-Pro-Gln-Leu-His-Leu-Arg-Phe-NH ₂	72	-31.9	26.9	Ala 1.0 Arg 2.0 Gly 1.0 Pro 0.99 Glu 1.0 Leu 2.1 His 1.0 Phe 0.97	1193.4 1192.8	0.22 0.40 0.13
VII	H-Ala-Arg-Gly-Pro-Gln-Leu-D-Arg-Leu-Arg-Phe-NH ₂	63	-15.8	25.8	Ala 0.98 Arg 3.0 Gly 1.05 Pro 1.0 Gln 0.99 Leu 2.1 Phe 0.98	1212.4 1212.8	0.30 0.48 0.16
VIII	H-Ala-Arg-Gly-Pro-Gln-Leu-Arg-Leu-Lys-Phe-NH ₂	76	24.6	26.1	Ala 0.99 Arg 2.1 Gly 1.04 Pro 1.1 Gln 0.98 Leu 2.1 Lys 1.0 Phe 0.97	1184.4 1183.8	0.21 0.35 0.17
IX	H-Ala-Arg-Gly-Pro-Gln-Leu-Arg-Leu-His-Phe-NH ₂	58	-16.4	26.7	Ala 1.0 Arg 2.0 Gly 1.05 Pro 1.0 Gln 0.98 Leu 2.1 His 1.0 Phe 0.97	1193.4 1193.7	0.24 0.50 0.14
X	H-Ala-Arg-Gly-Pro-Gln-Leu-Arg-Leu-D-Arg-Phe-NH ₂	71	-28.7	25.3	Ala 1.0 Arg 3.1 Gly 1.0 Pro 1.1 Gln 0.99 Leu 2.1 Phe 1.0	1212.4 1211.9	0.30 0.52 0.16
XI	H-Ala-Lys-Gly-Pro-Gln-Leu-Lys-Leu-Lys-Phe-NH ₂	38	-22.6	23.9	Ala 1.0 Lys 3.1 Gly 1.04 Pro 1.1 Gln 0.99 Leu 2.0 Phe 0.97	1128.4 1127.8	0.19 0.45 0.09
XII	H-Ala-Orn-Gly-Pro-Gln-Leu-Orn-Leu-Orn-Phe-NH ₂	33	-37.9	22.4	Ala 0.99 Gly 1.0 Pro 1.1 Gln 0.97 Leu 2.1 Phe 1.0	1086.3 1086.6	0.18 0.56 0.12

^a HPLC on C18 column (VYDAC) 250 x 4,6 mm; linear gradient: 0-80% in 60 min (80% acetonitrile in water + 0.1% TFA).

^b TLC on silica gel plates (Merck), eluents: X = n-butanol:Ac-OH:water (4:1:5), Y = n-butanol:pyridine:Ac-OH:water(30:20:6:24),

W = n-butanol:Ac-OH:ethyl acetate:water (1:1:1:1).

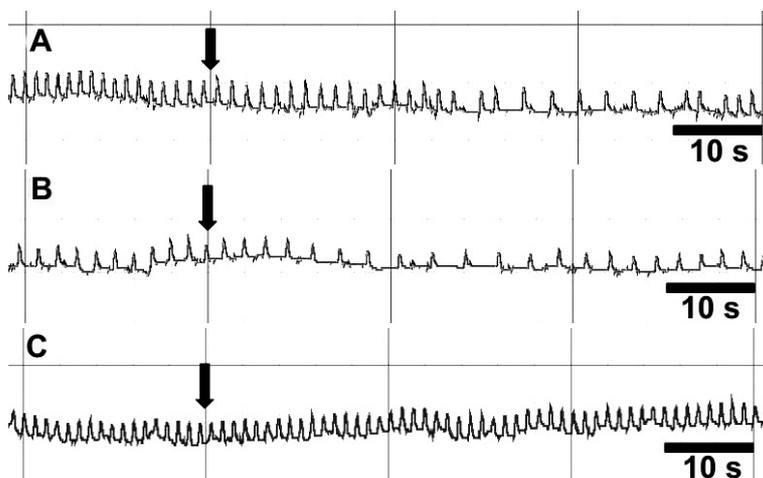


Figure 1. Myograms displaying typical responses of the contractile activity of the *Z. atratus* heart to 1×10^{-6} M of Led-NPF-I (A), and 1×10^{-6} M [His²-Led-NPF-I] (B) and [His⁷]-Led-NPF-I (C). Peptide application is indicated by an arrow.

In structure-activity relationship studies we compared cardioinhibitory action of the Led-NPF-I and its eleven analogues with Arg residues replaced by another basic amino acid. Replacement of Arg in position 2 with Lys (II), His (III) or D-Arg isomer (IV) seemed to enhance cardioinhibitory activity in tests with 10^{-9} M concentrations (Figure 2, panel A). Substitution of Arg⁷ by Lys (V) or D-Arg (VII), or of Arg⁹ by His (IX) or D-Arg (X), also rather increased than decreased the inhibitory activity. However, modification of the Led-NPF-I molecule by replacement in Arg at position 7 with His (VI) or at position 9 with Lys (VIII) led to crucial structural changes for biological function and caused a loss of cardioinhibitory activity by these analogues (Figure 2, panel B).

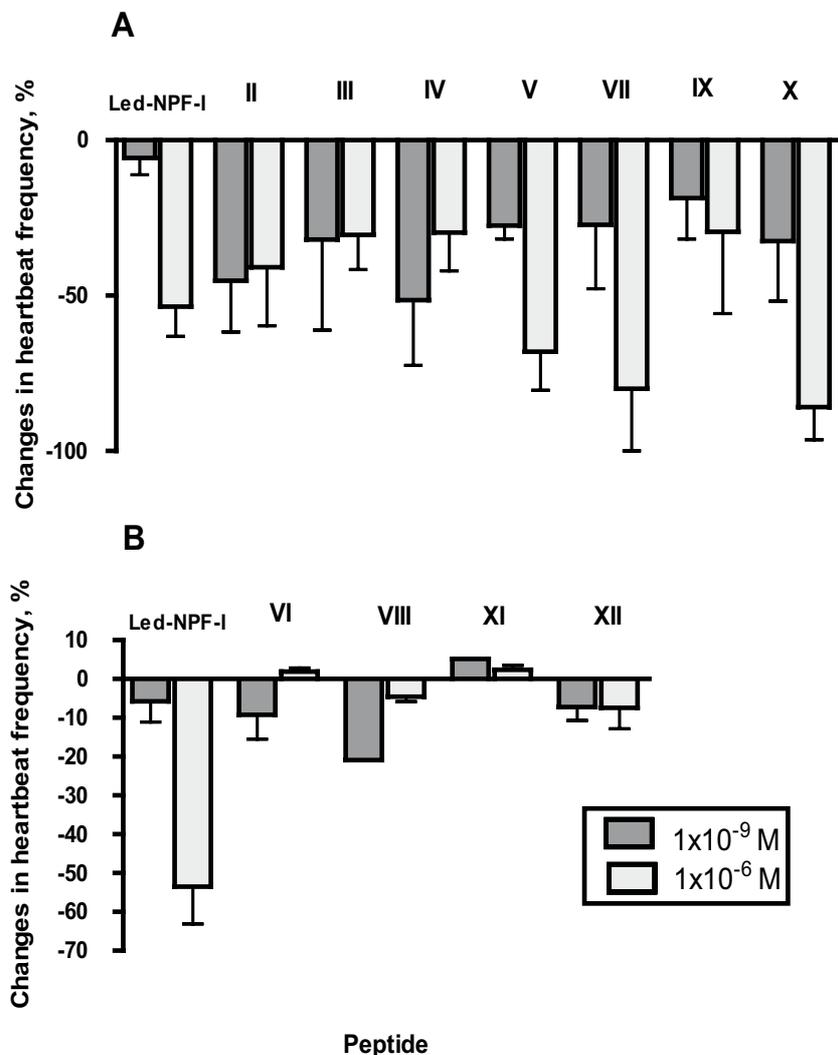


Figure 2. Cardioinhibitory activity of Led-NPF-I (I) and its analogues [Lys² – (II), [His² – (III), [D-Arg² – (IV), [Lys⁷ – (V), [His⁷ – (VI), [D-Arg⁷ – (VII), [Lys⁹ – (VIII), [His⁹ – (IX), [D-Arg⁹ – (X), [Lys^{2,7,9} – (XI) and [Orn^{2,7,9} – Led-NPF-I at lower (10⁻⁹ M) and higher (10⁻⁶ M) concentrations in the *Zophobas* heart bioassay. Panel A – active analogues. Panel B – inactive analogues. Mean values ±SEM are given at least three experiments.

All active analogues with a single modified Arg residues (II-V, VII, IX-X) at the low concentration (10^{-9} M), and also some of the analogues (V, VII, X) at higher concentrations (10^{-6} M) exhibited stronger cardioinhibitory activity than the native peptide. However, analogues of Led-NPF-I obtained by simultaneous replacement of Arg residue at all three positions 2, 7 and 9 with Lys (XI) or Orn (XII) resulted in a complete loss of cardioinhibitory activity. It was previously observed that simultaneous substitution in the Led-NPF-I molecule of all Arg residues with histidine, another basic amino acid, caused also a decline of cardioinhibitory activity of the analogue in *Z. atratus* [14]. These results suggests that replacement of Arg residues at all three positions represents a serious structural modification that is too severe to be tolerated by the putative receptor such that cardioinhibitory function of the native peptide may not to be preserved.

In *D. melanogaster*, it was shown that a sNPF G-coupled receptor is preferentially activated by sNPFs [17]. Only peptides with the typical RX_1RX_2 amide C-terminal sequence, such as the short NPFs of *D. melanogaster* or *S. gregaria*, were able to elicit a calcium response for the sNPF receptor expressing mammalian Chinese hamster ovary cells. The authors suggested that the receptor specificity is determined by the Arg residue in position 4 (counted back from the C-terminal end). An aromatic residue, such as Tyr or Phe, in this position, which is typical for the FMRFamide-related peptides, may prevent the peptide from binding to the receptor and hence eliciting a response. Garczyński et al. [18] observed that the sNPFs of *D. melanogaster* differ in their interactions with the sNPF receptor Drm-NPFR76R, as analyzed directly by radioreceptor assay. Binding assays revealed that longer Drm-sNPF-2 (Arg-Ser-Pro-Ser-Leu-Arg-Leu-Arg-Phe-amide), comprised of nine amino acids, was clearly more potent than shorter ones of eight (Drm-sNPF-1; Ser-Pro-Ser-Leu-Arg-Leu-Arg-Phe-amide) or six amino acids (Drm-sNPF-4; Pro-Gln-Arg-Leu-Arg-Trp-amide). These results were consistent with the structural activity pattern found for binding of *A. gambiae* sNPF peptides to Ang-sNPF receptor [19].

In conclusion, our studies shown that in the primary structure of the amino acid chain of the Led-NPF-I molecule Arg residue at position 2 is not as critical for cardioinhibitory function as the remaining two Arg residues at positions 7 and 9 that seem to play a crucial role in the preservation of peptide function during its interaction with the receptor on the *Z. atratus* myocardium.

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