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Effects of Neonatal Administration of Non-Opiate Analogues of Leu-Enkephalin to Heart Tissue Homeostasis of Prepubertal Albino Rats Exposed to Hypoxia

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Hypobaric hypoxia (pO_2 65 mm Hg, duration 4 h) induced a significant increase in the number of cardiomyocytes expressing p53, beclin-1, endothelial NO synthase and accumulation and degranulation of mast cells in the epicardium in hearts of prepubertal female rats (age 45-47 days); the number of cardiomyocytes with nucleoli decreased, while the number of single-nucleolar cardiomyocytes increased after this exposure. Five-fold administration of non-opiate analogue of leu-enkephalin (NALE peptide: Phe–D-Ala–Gly–Phe–Leu–Arg; 100 μg/kg) during the neonatal period reduced the severity of the post-hypoxic changes in the heart. Neonatal administration of NALE (100 μg/kg) against the background of NO synthase blockade with L-NAME (50 mg/kg) did not abolish the cardioprotective effects of the peptide. A similar correction of posthypoxic changes in the heart was observed after neonatal administration of original peptide G (Phe–D-Ala–Gly–Phe–Leu–Gly; 100 μg/kg). Thus, NO synthase–NO system and C-terminal amino acid Arg in the molecule of non-opiate analogue of leu-enkephalin are not required for the cardioprotective effects of peptides. Non-opiate leu-enkephalin analogs, peptides NALE and G, can be considered as promising substances for increasing heart resistance to hypoxia during later age periods.

Key Words: *leu-enkephalin analogues; cardiomyocytes; acute hypoxia; mast cells; apoptosis*

Improving heart resistance to hypoxia is one of the most important problems in experimental medicine. The agonists of opioid receptors can alleviate the consequences of ischemia/reperfusion damage to the myocardium [1]. Endogenous opioid peptides are considered to be the triggers of hypoxic preconditioning in heart [9]. In our previous studies, the effect of neonatal administration of the NALE peptide (a non-opiate analogue of leu-enkephalin) for the correction of the cardiac consequences of antenatal hypoxia was shown [13], which can serve as the rationale for designing new cardioprotective drugs for pediatric practice from this substance. The effects and mechanisms of the influence of neonatal administration of non-opiate analogue of leu-enkephalin on myocardial resistance to hypoxia during later age periods are most interesting questions.

The aim of the present study was to analyze the influence of acute hypoxia on the heart of prepubertal albino rats received leu-enkephalin analogues exhibiting no affinity to opioid receptors during the neonatal period.

MATERIALS AND METHODS

The experiments were performed on female albino Wistar rats (n=62). The experiments were performed and the animals were kept according to GOST 33216-

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2014 Guidelines for Accommodation and Care of Animals. Species-Specific Provisions for Laboratory Rodents and Rabbits. The study was approved by the Ethical Committee of the Far Eastern State Medical University.

Neonatal rat pups were divided by the litter division method into 5 experimental groups. Group 1 animals (control; *n*=12) daily received intraperitoneal injection of 0.1 ml of isotonic NaCl from the second to the sixth postnatal days; group 2 rats (hypoxia; *n*=12) daily received intraperitoneal injection of 0.1 ml of isotonic NaCl from the second to the sixth postnatal days and were exposed to hypoxia at the age of 45 days; group 3 rats (NALE+hypoxia; *n*=13) received intraperitoneal injections of non-opiate analog of leu-enkephalin — NALE peptide (Phe–D-Ala–Gly–Phe–Leu–Arg; Almabion) in a dose of 100 μg/kg in 0.1 ml isotonic NaCl on days 2-6 of life and subjected to hypoxia on day 45 of life; group 4 animals (NALE+L-NAME+hypoxia; *n*=12) were subjected to the same manipulations as group 3 animals and additionally received 50 mg/kg non-selective NO synthase inhibitor L-NAME (Sigma-Aldrich) in 0.1 ml isotonic NaCl on postnatal days 2-6; group 5 rats (G peptide+hypoxia; *n*=13) received daily intraperitoneal injection of original peptide Phe–D-Ala–Gly–Phe–Leu–Gly (Almabion) in dose 100 μg/kg in 0.1 ml isotonic NaCl on days 2-6 of life and subjected to the hypoxic exposure at the age of 45 days.

The NALE and G peptides are structural analogues of leu-enkephalin lacking affinity to opiate receptors due to substitution of Tyr for Phe in the first position of the amino acid chain. Hypoxic exposure (pO_2 65 mm Hg, duration 4 h) was modeled in a hypobaric chamber.

The animals were euthanized at the age of 47 days (48 h after hypoxic exposure) by rapid decapitation under chloroform vapor anesthesia. We used female rats because they are characterized by greater reactivity to hypoxia than males [2]. The use of prepubertal rats avoids methodological problems associated with estrus cycle.

The body weight (g), absolute (mg), and relative (heart weight to body weight ratio in %) heart weight were determined. The heart was subjected to histological processing: the tissue was fixed in neutral 10% formaldehyde and histotopographic paraffin sections were prepared.

In sections stained with silver nitrate by the AgNOR method modified by D. E. Korzhevsky (1990). To evaluate the condition of cell stress and indirectly assess protein-synthetic activity of cardiomyocytes (CMC), the number of nucleoli in the nuclei in the subendocardial zone of the left and right ventricles was estimated (at least 200 CMC were examined in each zone) [3].

Immunohistochemical detection of CMC stained with monoclonal antibodies to the cell alteration and apoptosis marker p53 (DO-7 clone, Leica Biosystems), polyclonal antibodies to autophagy marker beclin-1 (Cloud-Clone Corp.), and polyclonal antibodies to endothelial NO synthase (eNOS; Affinity Biosciences) was made using Novolink Polymer Detection System (Leica Biosystems) according to the manufacturer protocol. The proportion of cells with brown staining was determined by light microscopy (×1000); at least 500 CMC were viewed.

For detection of mast cells (MC), the histotopographic sections of the heart were stained with basic brown by the method proposed by M. G. Shubich (1958); the nuclei were poststained with Ehrlich's hematoxylin. In the epicardium of the left ventricle, the total number of MC, number of MC containing granules and degranulated MC per 1 mm^2 section area were determined.

Statistical processing of the results was performed using Statistica 6.0 software (StatSoft, Inc.). After testing for normality of data distribution, *M*±*SEM* were calculated. The significance of differences was evaluated by the Student's *t* test. The intergroup differences were considered statistically significant at $p<0.05$.

RESULTS

Hypoxic exposure had no effect on gravimetric parameters of the heart in prepubertal albino female rats, but significant changes in the heart tissue were observed. Analysis of CMC nucleoli in group 2 animals (hypoxia) showed a significant (almost 2-fold) decrease in the number of CMC nuclei containing ⩾4 nucleoli in the myocardium of the right ventricle (Fig. 1) and a significant (by 26.3%) increase in the proportion of CMC containing one nucleolus in the absence of significant decrease in the mean number of CMC nucleoli. These changes indirectly show a decline in protein-synthetic activity and cellular stress in CMC after hypoxic exposure [3].

The state of cellular stress in CMC in animals of this experimental group is confirmed by a significant increase in the number of $p53^+$ CMC (by 42.9 and 45.7% in left and right ventricles, respectively; Table 1). The considerable increase in the content $p53^+$ CMC number after hypoxic exposure is in line with published data: stabilization of HIF-1α under hypoxic conditions leads to accumulation of p53 protein, which can induce CMC apoptosis [6]. We also observed activation of autophagy in the myocardium: the proportion of beclin-1^+ CMC in left and right ventricles was increased by 50.3 and 43.5%, respectively (Table 1). Enhanced CMC autophagy is typical of hypoxic heart alteration [4].

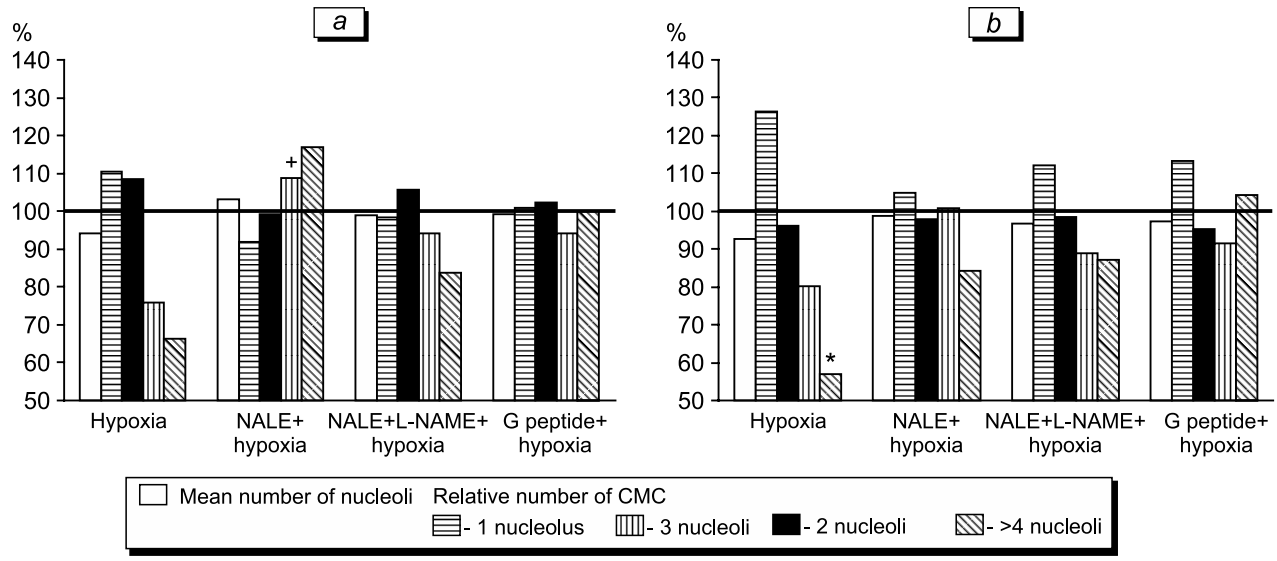

Fig. 1. Number of nucleoli in CMC nuclei and number of CMC with different numbers of nucleoli in the subendocardial zone of the myocardium of the left (*a*) and right (*b*) ventricles in rats of different groups. $p<0.05$ in comparison with *control (100%), $^{+}$hypoxia.

In animals exposed to hypoxia, a significant (by 2 times) elevation of the relative content of $eNOS^{+}$ CMC was revealed in the myocardium of the left and right ventricles (Table 2). eNOS activation in 48 h after hypoxia is possibly a compensatory mechanism of CMC protection from ischemia/reperfusion [8]. However, the excess of NO can aggravate the oxidative stress in the myocardium via generation of peroxynitrite ($ONOO^{-}$) [5].

Hypoxic exposure induced a significant increase in MC content in the epicardium of the left ventricle (Fig. 2). The total number MC per 1 mm^2 section area increased by 38.3% and the number of degranulating MC increased by 7 times in comparison with the control. The increase of MC number [12] and stimulation of MC degranulation in the heart during ischemia was previously reported [7]. It is known that MC degranulation leads to the release of proinflammatory factors: TNFα, histamine, and chymase. MC chymase is a stimulant of local angiotensin-2 production in the heart [12] and plays an important role in the negative effects of ischemia/reperfusion damage to the myocardium [10].

Repeated (5-fold, on days 2-6 of life) administration of NALE peptide to newborn animals modified the reaction of the heart in prepubertal albino rats to acute hypobaric hypoxia. In group 3 animals (NALE+hypoxia), the proportion of CMC with different

TABLE 1. Relative Number of $p53^{+}$ and Beclin-1^{+} Cardiomyocytes in the Myocardium of Albino Rats of Experimental Groups (%; *M±SEM*)

Group	Left ventricle		Right ventricle	
	$p53^{+}$	beclin-1^{+}	$p53^{+}$	beclin-1^{+}
Control (group 1)	5.74±0.54	16.17±0.80	5.69±0.38	17.51±0.89
Hypoxia (group 2)	8.20±0.75	24.31±1.14	8.29±0.78	25.13±1.09
	*p=0.02	*p=0.0001	*p=0.01	*p=0.0002
NALE+hypoxia (group 3)	6.54±0.58	20.89±1.01	7.63±0.35	21.26±0.64
		*p=0.0033	*p=0.003	*p=0.005
		^{+}p=0.044		^{+}p=0.009
NALE+L-NAME+hypoxia (group 4)	7.47±0.69	23.44±0.64	8.07±0.70	22.40±1.17
		*p=0.000005	*p=0.015	*p=0.007
G peptide+hypoxia (group 5)	6.26±0.32	16.84±1.89	6.60±0.51	16.98±2.48
	^{+}p=0.02	^{+}p=0.01		^{+}p=0.01

Note. Significant differences from *group 1, $^{+}$group 2.

numbers of nucleoli did not differ from the control parameters (Fig. 1). The number of p53$^+$ CMC remained higher (by 34.1%) than in the control group in the right ventricle, but did not differ from control in the left ventricle. The number of beclin-1$^+$ CMC in this experimental group significantly surpassed the control level (by 29.2 and 24.0% in the left and right ventricles, respectively), but was significantly lower than in group 2 (by 14.1 and 15.4% in the left and right ventricles, respectively; Table 1). The total number of MC and the content of granule-containing MC in the epicardium in group 3 rats did not differ from the control (Fig. 2). The number of degranulating MC was decreased by 35.7% in comparison with group 2 (Fig. 2). The number of eNOS$^+$ CMC in animals treated with NALE during the neonatal period was significantly higher (by 54.8%) after hypoxic exposure in comparison with control parameter, but was lower by 17.4% than in group 2 (Table 2).

Thus, neonatal administration of NALE peptide significantly reduced the severity of posthypoxic changes in the myocardium of prepubertal rats. The cardioprotective effect of neonatal administration of NALE peptide in acute hypoxia during later periods of life is described for the first time.

The presence of Arg in the NALE structure can imply the involvement of NO system in the realization of its effects. To test this hypothesis, NALE was administered to neonatal rats against the background of NO synthase blockade with non-selective inhibitor L-NAME (NALE+L-NAME+hypoxia; group 4). The blockade of NO synthesis did not change the positive effect of NALE on the nucleolar apparatus of CMC (Fig. 1) and the number of p53$^+$ CMC in the left ventricle (Table 1). The content of eNOS$^+$ CMC in this

TABLE 2. Relative Number of eNOS$^+$ Cardiomyocytes in the Myocardium of Albino Rats of Experimental Groups (*M*±*SEM*)

Group	Left ventricle, %	Right ventricle, %
Control (group 1)	10.83±0.96	11.20±0.91
Hypoxia (group 2)	20.29±1.32	20.96±1.60
	*p=0.00009	*p=0.0002
NALE+hypoxia (group 3)	16.77±1.10	16.31±1.61
	*p=0.0015	*p=0.017
NALE+L-NAME+ hypoxia (group 4)	16.16±1.24	16.40±1.17
	*p=0.006	*p=0.0008
	^+p=0.04	^+p=0.017
G peptide+hypoxia (group 5)	13.48±1.38	13.01±1.15
	^+p=0.004	^+p=0.0009

Note. Significant differences from *group 1, $^+$group 2.

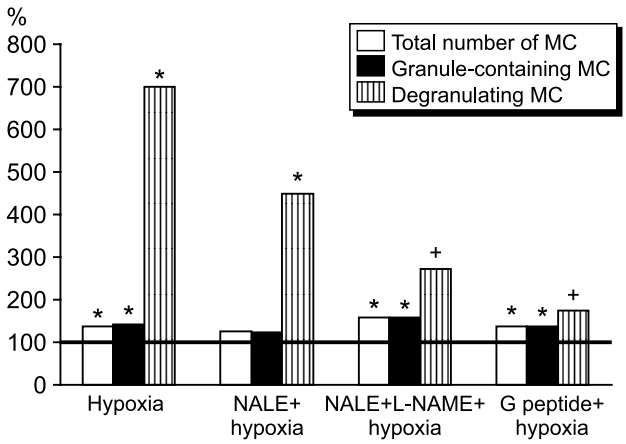

Fig. 2. Number of MC in the epicardium of the left ventricle in rats of different groups. p<0.05 in comparison with *control (100%), $^+$hypoxia.

experimental group was similar to that in group 3 (Table 2). However, the number of beclin-1$^+$ CMC (Table 1) and the content of MC in the epicardium (total number and the number of granule-containing MC) in this group did not significantly differ from those in group 2 (Fig. 2). The quantity of MC degranulations in this group was significantly lower, than in hypoxia group and had no differences from the control (Fig. 2). Thus, the animals received NALE peptide and NO synthase inhibitor L-NAME during the neonatal period demonstrated ambiguous changes in the cardiac response to hypoxic exposure. Hence, we cannot explain the cardioprotective effects of the NALE peptide exclusively by its interaction with the NO system.

For additional analysis of the role of Arg residue in the realization of the delayed cardioprotective effect of non-opiate analogue of leu-enkephalin, we studied the effect of neonatal administration of the modified non-opiate analogue of leu-enkephalin G peptide (substitution of C-terminal amino acid Arg for Gly). The parameters of the CMC nucleolar apparatus in group 5 animals (G peptide+hypoxia) did not significantly differ from those in the controls and group 3 rats (Fig. 1). In animals receiving G protein during the neonatal period, hypoxia did not change the number of p53$^+$, beclin-1$^+$, and eNOS$^+$ CMC (Tables 1 and 2). In the epicardium of these animals, a significant increase in the total number of MC and the number of granule-containing MC was observed, while the number of degranulating MC did not differ from the control. Thus, administration of original G peptide during the neonatal period could cause a pronounced cardioprotective effect under conditions of hypoxic exposure during later periods of life. Thus, the cardioprotective effect of non-opiate analogues of leu-enkephalin is not associated with the presence of Arg residue in the amino acid chain.

The results of our experiments showed that neonatal administration of peptides, non-opiate analogues of leu-enkephalin, can protect the heart from hypoxic damage during later age periods. Considering similar amino acid sequences of the examined peptides with N-terminal structure of nociceptin [14], the mechanisms of delayed cardioprotection after neonatal administration of non-opiate analogues of leu-enkephalin can be associated with epigenetic influence through NOR (ORL1)-receptor or other atypical opioid receptors [11].

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