EXPERIMENTAL BIOLOGY

PREVENTION OF DISTURBANCES INDUCED IN THE LIVER OF PROGENY OF ALBINO RATS BY PRENATAL HYPOXIA WITH THE AID OF A NONOPIATE LEU-ENKEPHALIN ANALOG

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In previous investigations the writers showed that if pregnant women are exposed to risk factors with a value of more than 9 points, DNA synthesis is disturbed and a threefold increase is found in the number of pathological mitoses (PM) in hepatocytes of the human fetus [11]. A model of this process was created by exposing albino rats to prenatal chronic hypoxia. Besides weakening of proliferative processes, reduction of the weight of the liver was observed, mainly due to an increase in area of the hematopoietic foci, whereas the area occupied by hepatocytes was considerably reduced [12]. It was also established that dalargin, a stable Leu-enkephalin analog, prevented the increase in the PM level and disturbance of DNA synthesis, induced by stress [9]. In the investigation described below, in order to prevent and correct morphological and biochemical disturbances arising in the liver of animals exposed to prenatal chronic hypoxia, we used another Leu-enkephalin analog, namely Phe-D-Ala-Glu-Phe-Leu-Arg, synthesized in the Laboratory of Peptide Chemistry, All-Union Cardiologic Scientific Center, Academy of Medical Sciences of the USSR. In its chemical structure it is close to dalargin (Tyr-D-Ala-Glu-Phe-Leu-Arg) but differs from it in not being a ligand of opiate receptors. Considering the important role of activation of lipid peroxidation (LPO) in disturbances of cell division [4], it was decided to study the role of free-radical processes in changes in proliferation in the liver of the newborn progeny of albino rats, exposed to chronic intrauterine hypoxia, and also the possibility of prenatal correction of these disturbances by means of a nonopiate analog of Leu-enkephalin.

EXPERIMENTAL METHOD

Experiments were carried out on 2-day-old newborn rats, divided into five groups. Group 1 consisted of animals not exposed to any procedures. Group 2 included rats whose mothers had been exposed to hypoxia from the 5th-6th day of pregnancy for 7 days by elevation to an altitude of 9000 m by the method described previously [12]. The mothers were given an injection of 0.1 ml of isotonic sodium chloride solution 30 min before the beginning of the session of hypoxia. Group 3 consisted of the progeny of animals which received an injection of the hexapeptide in a dose of 10 μg/kg body weight 30 min before the beginning of the session of hypoxia. To compare the efficacy of the action of the hexapeptide on LPO and on the morphological parameters, in group 4 the pharmacopeal preparation α-tocopherol was used, and was injected in the same way as the hexapeptide but in a dose of 10 mg/kg. Group 5 comprised young rats whose mothers had received the hexapeptide in a dose of 10 μg/kg for 7 days without undergoing any sessions of hypoxia. The study of the effect of α-tocopherol on the morphological and functional parameters of the progeny was not among the aims of the present investigation, On the 2nd day after birth the rats were weighed, humanely killed, and the absolute mass of the liver

*Deceased.

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TABLE 1. Effect of Prenatal Correction with Phe-D-Ala-Glu-Phe-Leu-Arg and α-Tocopherol on Body Weight and Morphometric Parameters of Newborn Progeny of Albino Rats Exposed to Hypoxia

Group of animals	Conditions	Body weight, g	Mass of liver, g	Mass of liver/ body weight	Relative area, %		
					hepatocytes	vessels	foci of hematopoiesis
	control	6,565±0,111	0,169±0,006	1/39	86,9±1,0	5,9±0,5	7,2±0,8
2	Hypoxia	5,618±0,182*	0,209±0,005*	1/27	78,8±1,1*	6,3±0,8	14,9±0,6*
3	Hypoxia + hexapeptide	6,644±0,119**	0,170±0,005**	1/39	84,5±1,1**	6,1±0,6	9,4±0,8*,**
4	Hypoxia + α-tocopherol	6,321±0,150**	0,176±0,004**	1/36	87,4±1,1**	5,7±0,8	6,9±0,8**,***
5	Intact control + hexapeptide	6,700±0,120**	0,173±0,003**	1/39	84,6±0,9*,**	8,6±0,4**	6,8±0,5**

Legend. Here and in Table 2, *) significant differences compared with group 1, **) compared with group 2, ***) significant differences between groups 3 and 4.

TABLE 2. Effect of Prenatal Correction by Phe-D-Ala-Glu-Phe-Leu-Arg and α-Tocopherol on Proliferative Processes and LPO and AOA System in Newborn Progeny of Albino Rats Exposed to Hypoxia

Group of animal	Conditions	MI, %	PM, %	ILN, %	LI	MDA, fluorescence units	LHP	AOA
							optical density units	
1	Intact control	12,7±0,7	6,7±0,6	10,2±0,5	33,8±1,5	311,0±39,0	3,1±0,4	1,6±0,2
2	Hypoxia	9,1±0,5*	17,6±0,5*	6,7±0,5*	26,5±1,3*	888,0±98,0*	2,8±0,3	0,8±0,1*
3	Hypoxia + hexapeptide	12,9±0,8**,***	8,9±0,8*,**,***	8,4±0,5*,**	32,3±1,9**	299,0±42,0**	2,7±0,4	1,4±0,2**
4	Hypoxia + α-tocopherol	9,3±0,7*	12,7±1,6*,**	7,8±0,3*	27,5±0,3*,**	263,0±32,0**	2,4±0,3	1,4±0,2**
5	Intact control + hexapeptide	12,2±1,0	7,0±0,8	9,7±0,6	29,6±0,9	289,5±46,7	3,3±0,4	1,6±0,3

was determined. Pieces of liver measuring 2 mm^3 were incubated in an ultrathermostat at 37°C in medium 199 containing ^{3}H-thymidine (5 μCi/ml, specific activity 84 Ci/mmole), Autoradiographs were prepared and the index of labeled nuclei (ILN, in %) and labeling intensity (LI) were determined by the method described previously [11]. In addition, to determine the mitotic index (MI, in promille) and the level of PM (in % of the total number of mitoses) pieces of liver were fixed in Carnoy's fluid. MI was determined on the basis of phase-differentiated counting of mitotic figures during examination of at least 3000 hepatocytes. To identify aberrant mitotic figures we used I. A. Alov's classification [3]. Systematic stereometric analysis was carried out by morphometry, using the fields method and the technique described in [1]. Antioxidative activity (AOA) was determined by the method in [7]. The concentration of lipid hydroperoxides (LHP) was estimated by the method in [6]. The LHP and AOA levels were estimated in optical density units per gram of lipids, determined by the method in [14], Malonic dialdehyde (MDA) was determined by the method in [13] in fluorescence units and calculated per gram of lipids. Altogether 372 newborn animals from 73 mother rats were used in the experiments. For statistical analysis of the results standard methods were used to calculate mean values and their deviations by the Fisher—Student method. Differences between means were taken to be significant at the $p \leq 0.05$ level.

EXPERIMENTAL RESULTS

Just as in previous investigations, prenatal chronic hypoxia caused reduction of the body weight of the newborn rats and an increase in weight of the liver, as a result of which the ratio of the weight of the liver to the total weight of the fetus increased. The liver lagged behind the gestation times because of intensification of its hematopoietic role during hypoxia. Evidence in support of this view was given by the twofold increase in area of the hematopoietic tissue, while the area of the parenchyma was reduced by 10% ($p \leq 0.05$) compared with the control (Table 1). These changes were largely

due to inhibition of proliferative processes in the hepatocytes: ILN, LI, and MI were all significantly reduced. In addition, the number of PM was increased and severe and lasting pathological forms appeared (bridges, multipolar mitoses).

Chronic intrauterine hypoxia led to intensification of LPO. This was shown by an increase in the MDA concentration in the neonatal rat liver by 2.7 times. The LHP content did not change significantly. This was evidently due to intensification of enzymic conversion of LHP into products of their subsequent transformation (Table 1). Besides activation of free-radical processes, there was a decrease in AOA, preventing the damaging action of free radicals. It can be tentatively suggested that activation of LPO and depression of AOA played a significant role in the disturbances of cell division of the hepatocytes in the present experiments. In the modern view excessive activation of LPO during hypoxia may lead to membrane damage, destruction of enzymes, and disturbance of DNA synthesis and cell division [5].

Administration of the hexapeptide prevented the development of prenatal hypotrophy: the body weight of the newborn rats did not differ from the control values. The mass of the liver was significantly reduced ($p < 0.01$) compared with the posthypoxic animals and did not differ from the control values. The ratio of the mass of the liver and the animals' body weight also corresponded to the analogous control values (Table 1). The hexapeptide prevented the development of functional immaturity of the liver relative to the time of gestation. This was expressed as a decrease in the area occupied by hematopoietic tissue by 1.6 times compared with this same parameter in the progeny of a mother which had not been given the hexapeptide before the session of hypoxia. Incidentally, administration of the hexapeptide did not cause complete normalization of this disturbance: the area of the hematopoietic foci was 1.3 times greater than in the control. The results of analysis of proliferative processes under these conditions shed light on the cause of the protective action of the hexapeptide. Although injection of the preparation did not fully restore ILN of the hepatocytes, this parameter was significantly higher than in the posthypoxic animals. LI, reflecting the velocity of DNA synthesis, was higher than the analogous parameter in posthypoxic animals and did not differ from the control values. It is important to note that the use of the hexapeptide led to a 50% reduction in the number of PM compared with that in the posthypoxic animals. However, this parameter was significantly (1.3 times) higher than the intact controls. A significant decrease was observed in the number of resistant pathological states such as bridges, and complete disappearance of tripolar mitoses were observed. The study of LPO in the liver shows that a definite part of the protective effect of the hexapeptide was due to weakening of peroxidation and to an increase in AOA.

The MDA concentration was only one-third as high as in the posthypoxic group ($p \leq 0.01$) and did not differ from values in the intact control. Meanwhile AOA showed a return to normal compared with the intact control, but it was increased by 1.7 times compared with AOA in the posthypoxic group.

A comparative study of the action of the hexapeptide and α-tocopherol on free-radical oxidative processes showed that the end results were identical. It must be remembered, however, that the dose of α-tocopherol was $2.3 \cdot 10^{-6}$ M, whereas the hexapeptide was given in a dose of $2.0 \cdot 10^{-9}$ M. It must be particularly noted that although the effects of the preparations studied in relation to the LPO and AOA system are identical, the hexapeptide had a stronger effect on proliferative processes. It lowered the PM level more effectively than α-tocopherol ($p \leq 0.05$). Elevation of the PM level is interpreted as an adequate parameter of the cytopathic effect [2]. The ability of the nonopiate Leu-enkephalin analog studied in these experiments to reduce the number of aberrant mitotic figures, in our view, signifies a cytoprotective action. It will be recalled that the opiate Leu-enkephalin analog dalargin [10] possesses marked cytoprotective properties. Injection of the hexapeptide into pregnant females not exposed to sessions of hypoxia did not lead to any changes in proliferative processes in the hepatocytes. The morphometric data (mass of the newborn infants and their liver, ratio between the areas of parenchyma, blood vessels, and hematopoietic foci) and the biochemical parameters (LHP, MDA, AOA) likewise were virtually indistinguishable from the analogous values in intact animals. In this connection it is worth noting that in previous investigations this hexapeptide had no effect on cell division in the corneal epithelium of albino rats, whereas opiate analogs stimulated proliferative processes [8].

Thus prenatal administration of the hexapeptide prevented intrauterine hypotrophy, functional immaturity of the liver, and cytogenetic disturbances induced by hypoxia and promoted normalization of cell division. An important role in the realization of these effects of the hexapeptide is evidently played by its ability to normalize free-radical oxidative processes and to enhance the antioxidative protection of the body.

The close similarity in the chemical structure of the peptide and natural Leu-enkephalin, and its high efficacy in small doses necessitate a comprehensive study of its biological activity.

LITERATURE CITED

1. G. G. Avtandilov, N. I. Yabluchanskii, and V. G. Gubenko, Systematic Stereometry in the Study of a Pathological Process [in Russian], Moscow (1981).
2. A. B. Avtsyn and V. A. Shafranov, Ultrasonic Bases of Cell Pathology [in Russian], Moscow (1979).
3. I. A. Alov, Cytophysiology and Pathology of Mitosis [in Russian], Moscow (1972).
4. E. B. Burlakova, A. V. Alesenko, E. M. Molochkina, et al., Bioantioxidants in Radiation Sickness and Malignant Growth [in Russian], Moscow (1975).
5. O. N. Voskresenskii and A. P. Levitskii, Vopr. Med. Khim., No. 5, 563 (1970).
6. V. B. Gavrilov and M. I. Mishkorudnaya, Lab. Delo, No. 3, 33 (1983).
7. A. P. Levitskii, L. M. Shafranov, and E. K. Gudkevich, Inventor's Certificate 11 656614, 1979, USSR.
8. T. D. Pan'kova and S. S. Timoshin, Byull. Éksp. Biol. Med., No. 7, 96 (1990).
9. S. S. Timoshin, N. I. Berezhnova, and S. I. Shvets, Byull. Éksp. Biol. Med., No. 2, 189 (1990).
10. M. I. Titov, V. A. Vinogradov, and Zh. D. Bespalova, Byull. Vses. Kardiol. Nauch. Tsent., No. 2, 72 (1985).
11. L. I. Utkina, Perinatal Pathology and Diseases of Infants [in Russian], Khabarovsk (1987), pp. 63-65.
12. L. I. Utkina and S. S. Timoshin, Byull. Éksp. Biol. Med., No. 11, 541 (1990).
13. T. N. Fedorova, T. S. Korshunova, and E. G. Larskii, Lab. Delo, No. 3, 25 (1983).
14. J. A. Knight, S. Anderson, and J. M. Rawle, Clin. Chem., **18**, 199 (1972).

HEPATOCYTES FROM THE LIVER OF MICE WITH EXPERIMENTAL POST-TOXIC CIRRHOSIS STIMULATE DNA SYNTHESIS IN HETEROKARYONS IN NUCLEI OF RESTING NIH 3T3 FIBROBLASTS

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Chronic inflammation and necrosis in the liver parenchyma in cirrhosis of varied etiology are accompanied by pathological morphological and functional changes caused by local or general proliferation of the hepatic connective tissue. Fibrosis of the liver not only prevents the course of regeneration in the parenchyma of the lobules, but also disturbs function of the vascular system of the liver, leading to portal hypertension and to further aggravation of the pathological process [2]. The question arises of the mechanisms of stimulation of proliferative activity of the fibroblasts of the connective-tissue basis of the liver in chronic inflammatory and degenerative processes, for knowledge of these mechanisms would enable ways of their specific blocking to be found.

Experiments with fusion of hepatocytes, obtained from the liver regenerating after partial hepatectomy, and also of cells obtained from embryonic liver (15-18-day embryos) with resting NIH 3T3 fibroblasts have shown considerable stimulation of DNA synthesis in heterokaryons in the fibroblast nuclei [3]. We also observed stimulation of DNA synthesis in mononuclear cells (monokaryons) both in fusion experiments and during combined culture of fibroblasts with these hepatocytes. These facts suggest that hepatocytes obtained from the actively proliferating liver form factors which can stimulate proliferation of cells of mesenchymal origin.

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