Effects of Amino Acid Combinations on the Development of Organotypic Culture of the Myocardium from Young and Old Rats

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Effects of 20 L-amino acids in concentration of 0.05 ng/ml on the development of myocardium of young (3-month-old) and old (24-month-old) rats in organotypic tissue culture were investigated. Stimulation of cell proliferation in the myocardium of young animals appeared under the influence of asparagine, histidine, serine, lysine, arginine, glutamic acid, and isoleucine. In the myocardium of old rats, proliferative effects remained only for two amino acids, lysine and arginine. Combinations of one stimulating and one inactive amino acids effectively increase the stimulating effect in both young and old rats. Modulating properties of amino acids and their combinations provide the basis for the synthesis of peptides regulating regenerative processes in the myocardium, particularly during aging.

Key Words: *organotypic tissue culture; amino acids; proliferation*

Reparative processes in tissues can be regulated by various cytokines and peptides through stimulation or suppression (apoptosis) of cell proliferation [2,4]. However, amino acids entering their composition may possess some regulating properties for the target tissues by themselves. It was noted in 1960-s that radiolabeled amino acids are more or less intensively accumulated in cultured tissues depending on the tissue type [9]. Experiments on tissue cultures of the intestinal mucosa, testes, and spleen showed that inhibitory effect of neutral amino acids increased with increasing the length of carbohydrate side chain. Proline suppressed the development of brain cortex and spleen [11]. The effects of coding amino acids on cellular processes are intensively studied during two recent decades. Analysis of specific and nonspecific resistance showed that lysine, arginine, glutamic and asparagine acids, and tryptophan exhibit different immunostimulating, phagocytosis-stimulating, and detoxicant properties. After subcutaneous administration to mice, lysine and arginine stimulated phagocytosis, but did not protect against toxic compounds. In addition, lysine had no effect on the immune response, while arginine suppressed it [1]. Examination of preimplantation pig embryos showed that amino acid consumption depended on the stage of embryo development. Stages from single-cell embryo to morula (days 0-4) are characterized by increased glutamine and threonine uptake, whereas blastocyst (day 6) is characterized by isoleucine, valine, phenylalanine, methionine, and arginine uptake [5]. It was shown on PC3 and DU145 androgen-independent prostate cancer cell strains that methionine, tyrosine, and phenylalanine deprivation produces an inhibiting effect with cell cycle arrest in G0/G1. Methionine deprivation increases apoptosis in PC3 cells, whereas tyrosine and phenylalanine deprivation increases apoptosis in DU145 cells [8]. A number of studies showed that glutamine-utilizing cells possess molecular mechanisms determining glutamine content and specific response to changes in extracellular glutamine. Thus,

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cell became more sensitive to Fas-mediated apoptosis when extracellular glutamine level decreased [12]. Addition of the spinal fluid enreached with glutamate from the patients with amyotrophic lateral sclerosis to the culture of brain neurons induced apoptosis. [13]. Leucine activated signal components triggering mRNA translation, stimulated protein synthesis in skeletal muscles of newborn pigs through activation of mTOR-receptors (mammalian target of rapamycin), and stimulated S6 kinase 1 (S6K1), eukaryotic initiation factor (eIF4E) [14]. Leucine increased mRNA translation and protein synthesis in rat myocardium under conditions of inhibition of this synthesis in acute ethanol poisoning [15]. There is evidence that signal pathways for amino acids may involve mTOR receptors and therefore control numerous components of translation, including initiation and elongation factors [10]. In this context, it seems important to evaluate the regulatory effects of all 20 essential and nonessential amino acids encoded by the genome and forming the basis of biological processes on the myocardium. This issue was not systematically investigated up to date. Organotypic culturing of tissue fragments is the most adequate approach for fast qualitative assessment of the effect directions of investigated biologically active compounds [2,3]. Advantages of tissue culture consist in the absence of nervous, humoral, and over influences, which are present in the body, and preserved "hierarchical" relationship between different cellular populations.

The objective of this study was screening of individual effects of 20 L-amino acids and their combinations on the development of organotypic culture of the myocardium in young and old rats.

TABLE 1. Effects of Amino Acid on AI (%) of Myocardium Explants from Young and Old Rats (*M±m*)

Amino acid	Age, months	
	3	24
Gly	-	-
Ala	-	-
Asn	**+19±2***	-9
His	**+31±5***	-
Lys	**+30±6***	**+22±5***
Ser	**+18±2***	-
Gln	-	-
Arg	**+29±5***	**+24±3***
Pro	-	-
Glu	**+35±4***	-
Asp	+6±5	-
Cys	+9±1	-
Tyr	-	+14±5
Val	-	-9±3
Thr	-	-
Met	-	-
Leu	+15±1	-
Ile	**+19±2***	-
Phe	-	-7±3
Trp	-8±1	-8±5

Note. Amino acids exerting stimulating influences on cell proliferation are marked in bold. Here and in Table 2: (–) – control level (0%). *$p<0.05$ in comparison with control AI.

MATERIALS AND METHODS

Organotypic culturing of the tissue was carried out according to previously described method [2,12]. We used 400 myocardium explants from 3- and 24-month-old Wistar rats. Tissue fragments were prepared under sterile conditions, divided into smaller parts approximately 1 mm^3 in size, and placed to collagen-coated Petri dishes. Culture medium contained 35% Eagle medium, 35% Hanks solution, 25% fetal bovine serum and 5% chicken embryo extract. The medium was supplemented with glucose (0.6%), insulin (0.5 U/ml), gentamycin (100 U/ml), and L-amino acids (Sigma): glycine (Gly), alanine (Ala), asparagine (Asn), histidine (His), lysine (Lys), serine (Ser), glutamine (Gln), arginine (Arg), proline (Pro), aspartic acid (Asp), glutamic acid (Glu), tyrosine (Tyr), cysteine (Cys), valine (Val), threonine (Thr), methionine (Met), leucine (Leu), isoleucine (Ile), phenylalanine (Phe), and tryptophan (Trp). Titration revealed that effective amino acid concentration is 0.05 ng/ml. Petri dishes were placed into a thermostat at 37°C and after 3 days were examined under a phase-contrast microscope. Area index (AI) was determined in conventional units as the ratio of the total explant area (including growth zone) to the central zone area. Microtelevision attachment for the microscope was used for explant visualization (10 series, MTH-13, Alfa-Telecome). PhotoM 1.2 software was used for calculation of explant AI. A total of 20-25 experimental explants and 20-23 control explants were used for each compound. Significance of differences between the control and experimental AI values was assessed using Student's *t*-test.

RESULTS

On day 1, spreading of the explants on the collagen coating with formation of the growth zone consisting

of proliferating and migrating cardiomyocytes with admixture of macrophages and fibroblasts was observed. Examination of the explants from young rat myocardium showed that medium supplementation with Asn, His, Lys, Ser, Glu, Arg, and Ile increased AI by 19-35% (Table 1). For instance, Glu increased AI by 35±4% (n=20, p<0.05) in comparison with the control (n=21), His by 31±5% (n=25, p<0.05) in comparison with the control (n=23). Leu produced a statistically insignificant AI increase. Other amino acids did not affect AI. Thus, unlike spleen tissue culture with high regeneration potential [2,3], none amino acid exerted proapoptotic effects on postmitotic myocardium tissue. The number of amino acids stimulating proliferation in myocardium explants from old rats decreased by 3.5 times: only Arg and Lys were active. Combinations of two stimulating amino acids produced no potentiating effect, which can be explained by the same transport pathways for these amino acids. However, combination of a stimulating amino acid with an inactive amino acid significantly potentiated the effect of stimulating amino acid. Thus, Lys alone increased AI by 30%, Trp statistically insignificantly decreased AI by 8%, but their combined administration resulted in significant stimulation of the growth zone and increased AI by 58%, what exceeds AI increment following isolated Lys administration by 28% (Table 2).

The following efficient amino acid combinations were found. Lys (AI stimulation by 30%) combined with Leu (statistically insignificant AI improvement by 15%) increased AI by 70±11% (n=22, p<0.05) in comparison with the control (n=23); Asn (AI stimulation by 19%) in combination with Leu — by 38±3% (n=21, p<0.05) in comparison with the control (n=23); Gln (AI stimulation by 29%) in combination with Val (AI at the control level) — by 56±4% (n=23, p<0,05) in comparison with the control (n=20); Asn (AI stimulation by 19%) in combination with Val (AI at the control level) — by 60±9% (n=24, p<0,05) in comparison with the control (n=21); Arg (AI stimulation by 29%) in combination with Val (AI at the control level) — by 47±3% (n=23, p<0.05) in comparison with the control (n=22); Ile (AI stimulation by 19%) in combination with Val (AI at the control level) — by 38±5% (n=25, p<0.05) in comparison with the control (n=20).

Thus, in the culture of young rat myocardium AI for combinations of amino acids exceeded AI for stimulating amino acid alone by 18-41%. In explants of old rat myocardium, AI for combination of amino acid exceeded AI of stimulating amino acid by 15-17% in comparison with the control (p<0.05) in two cases: Lys (AI stimulation by 22% for Lys alone) with Leu (AI at the control level for Leu alone) — AI increased by 39±7% (n=21, p<0.05) in comparison with the control (n=22), and Arg (AI stimulation by 24% for Arg alone) with Val (AI at the control level for Val alone) — AI increased by 44±5% (n=24, p<0.05) in comparison with the control (n=23).

On the basis of obtained data one may conclude, that combinations of one stimulating and one inactive amino acids were effective in terms of increase of stimulating effect in young and old rat myocardium. In this case, a dynamic balance between proliferation and apoptosis necessary for tissue development is maintained. Effective concentration for amino acids was 0.05 ng/ml (10^{-12} M range), which can be associated with the effect of ultralow doses, which is the subject of increased interest at the moment. In our experiments, the effects of amino acids were observed in the presence of some amino acids in the medium, which confirms the concept that minor shifts of concentration, rather than absolute concentrations of bio-

TABLE 2. Effects of Amino Acid Combinations on AI (%) of Myocardium Explants from Young and Old Rats (M±m)

Amino acid combination	3 months		24 months	
	AI	Increase in AI with combination of amino acids AI of stimulating amino acid	AI	Increase in AI with combination of amino acids AI of stimulating amino acid
Lys+Trp	58±11*	28%	24±3*	2%
Lys+Leu	70±11*	40%	39±7*	17%
Asn+Val	60±9*	41%	–	
Asn+Leu	38±3*	19%	–	
Arg+Val	47±3*	18%	44±5*	15%
Ile+Val	38±5*	19%	–	
Glu+Val	56±4*	21%	–	

logically active compounds are important [2,3,13]. In old animals, both the number of stimulating amino acids and the magnitude of their effects decreased. The modulating properties of amino acids and their combinations provide the basis for the synthesis of peptides regulating regeneration processes in the myocardium, particularly during aging.

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