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Activities of Some Caspase Cascade Enzymes and Myocardial Contractility in Experimental Left Ventricular Focal Ischemia

M. L. Blagonravov, M. M. Azova, M. V. Onufriev*, and V. A. Frolov

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Focal left ventricular ischemia was modeled in male Chinchilla rabbits. Activities of caspase-3 and caspase-8 in the left and right ventricular myocardium and myocardial contractility were studied after 1, 3, and 5 days. Caspase-3 activity increased significantly in the left ventricular peri-infarction zone and right ventricular myocardium, while caspase-8 activity did not differ from the control. Left ventricular contractility decreased significantly and the hemodynamic load of the right ventricle sharply increased. These results attest to induction of the internal (mitochondrial) pathway of apoptosis in myocardial cells most likely caused by left ventricular hypoxia and right ventricular overload.

Key Words: *apoptosis; ischemia; caspase; myocardium; contractile function*

Injury of the left ventricle (LV) in acute myocardial infarction is not confined to the zone with macroscopic signs of necrosis. A more extensive area of the myocardium is ischemized, as a result of which the so-called peri-infarction zone is formed with few sites of necrotic cell death; however, most cardiomyocytes (CMC) remain viable for some time. This status, so called "hibernation" or "stunning", is paralleled by triggering of genetic mechanisms of cellular molecular adaptation to continuing ischemia [10]. Clear-cut signs of inflammatory reaction develop in the peri-infarction and even distant intact sites of LV wall [4]. Apoptosis of CMC is induced in the myocardial prenecrotic zone [3,13]. Right ventricular (RV) CMC are also subjected to apoptosis in LV ischemia [5]. On the other hand, the mechanisms stimulating apoptosis in myocardial cells of LV and RV remain not quite clear.

We studied activity of some caspase cascade enzymes and LV and RV contractility in focal LV ischemia.

MATERIALS AND METHODS

The study was carried out on male Chinchilla rabbits (3.0-3.5 kg) in two parallel series, each on 20 animals divided into 4 groups. Group 1 (control) consisted of intact rabbits and three experimental groups consisted of rabbits with focal LV ischemia tested on days 1, 3, and 5, respectively. The animals were kept and handled in accordance with the Order No. 755 of the Ministry of Health of the USSR of August 12, 1977.

Focal LV ischemia was modeled by ligation of the descending left coronary artery between its middle and lower thirds in a surgical operation under general anesthesia.

Department of General Pathology and Pathophysiology, University of Peoples' Friendship of Russia; *Institute of Higher Nervous Activity and Neurophysiology, Russian Academy of Sciences, Moscow, Russia. ***Address for correspondence:*** blagonravovm@mail.ru. M. L. Blagonravov

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In experimental series I, the biochemical mechanisms responsible for myocardial cell apoptosis were studied. Activities of effector caspase-3 and initiator caspase-8 were measured in the LV peri-infarction zone and in RV myocardium. Activated caspases play the key role in the realization of the final stages of programmed cell death due to their capacity to cleave certain protein substrates. The appearance of products of this specific proteolysis is an important marker of apoptosis [1]. Caspase-3 is the final enzyme of the caspase cascade responsible for programmed cell death during realization of apoptogenic mechanisms through the internal and external signal routes. Activity of this enzyme reflects changes in apoptosis intensity throughout the process, and hence, it was evaluated during each term of the experiment. The initiator caspase-8 is triggered by activation of specific membrane receptors (Fas, TNF, DR, *etc.*) by various trigger molecules (TNF, lymphotoxins, *etc.*) [15], this reflecting just the involvement of the external signal route of apoptosis realization. Hence, measurements of caspase-8 were carried out only when caspase-3 activity reached the peak.

The thorax was opened in narcotized animals and the heart was removed. Specimens of LV and RV myocardium (200-250 mg) were isolated. The LV tissue was collected from sites of viable myocardium bordering the necrotic zone. Myocardial tissue (LV and RV separately) was fragmented in a WiseTis HG-15 homogenizer (8 mm rotor, 4500 rpm). Isolation medium (20 mM HEPES pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT) was used, to which protease inhibitor cocktail was added (104 mM AEBSF, 0.08 mM aprotinin, 1.5 mM pepstatin A, 2 mM leupeptin, 4 mM bestatin, and 1.4 mM E-64) in 100:1 proportion (all reagents from Sigma). Homogeneous suspensions were centrifuged on an Heraeus fresco 17 microcentrifuge (Thermo Electron LED GMBH) at 15,000*g* for 30 min at 4°C. The studies were carried out using equipment of the common use center of University of Peoples' Friendship created within the framework of realization of Priority National Project "Education". The supernatants were used for evaluation of caspase-3 and caspase-8 activities.

Activity of caspase-3 was measured by the colorimetric method by the rate of cleavage of synthetic substrate Ac-DEVD-pNA (N-acetyl-Asp-Glu-Val-Asp-nitroaniline, Sigma). The supernatant was incubated in 96-well microplates (95 min, 37°C) in reaction buffer (20 mM HEPES pH 7.4, 2 mM EDTA, 5 mM DTT, 0.1% CHAPS) in two parallel samples, one with 20 nM Ac-DEVD-pNA, the other with 20 nM Ac-DEVD-pNA and 2 nM Ac-DEVD-CHO specific caspase-3 inhibitor. Optical density was recorded every 10 min on a Sunrise EIA reader (Tecan) at λ=405 nm. Activity of caspase-3 was calculated by the difference of substrate cleavage rates in samples without and with the inhibitor with consideration for reference pNA optical density calibration curve.

Activity of caspase-8 was measured by the colorimetric method by the rate of Ac-IETD-pNA (N-acetyl-Ile-Glu-Thre-Asp-nitroaniline, Sigma) synthetic substrate cleavage. The supernatant was incubated in 96-well microplates at 37°C in a reaction buffer containing 20 mM HEPES pH 7.4, 2 mM EDTA, 5 mM DTT, 0.1% CHAPS, and 5% sucrose in two parallel samples, one with 20 nM Ac-IETD-pNA, the other with 20 nM Ac-IETD-pNA and 0.05 nM Ac-IETD-CHO, a specific caspase-8 inhibitor. Optical density was recorded every 10 min throughout 1 h on a Sunrise EIA reader (Tecan) at λ=405 nm. Activity of caspase-8 was also calculated by the difference of substrate cleavage rates in samples without and with the inhibitor with consideration for reference pNA optical density calibration curve.

The contractility of LV and RV was studied in experimental series II. The thorax was opened in narcotized animals and forced ventilation of the lungs was directly switched on. The real hemodynamic intraventricular pressure (RIP) and maximum developing intraventricular pressure (MIP) in LV and RV were measured using Micard programmed complex (PC-compatible analog digital transformer with electromanometric pickups) during 5-sec occlusion of the ascending aorta.

The data were processed using software created at Department of Pathology and Pathophysiology of University of Peoples' Friendship and Biostat software. The results were analyzed using Student's *t* test (the differences in the means were considered significant at $p<0.05$). The relationships between the processes and phenomena were detected by analysis of correlations.

RESULTS

By the end of day 1, activity of caspase-3 in LV myocardium increased significantly (Table 1). On day 3,

TABLE 1. Activities of Caspase-3 and Caspase-8 in LV Myocardium in Its Focal Ischemia (*M±m*)

Group		Caspase-3	Caspase-8
Control		0.1±0.01	0.19±0.05
Experimental	day 1	0.23±0.03*	Not measured
	day 3	0.26±0.04*	0.27±0.04
	day 5	0.15±0.04	Not measured

Note. Here and in Tables 2, 3: *$p\leq0.05$ compared to the control.

the parameter reached the maximum level and on day 5 virtually returned to the initial level. These data indicate stimulation of enzymatic mechanisms responsible for apoptosis in LV myocardium during the acute period of its ischemic injury.

Activity of caspase-8 measured on day 3 just tended to increase, its values did not virtually differ from the control. Hence, the caspase cascade in LV ischemia seemed to be induced by only the internal (mitochondrial) mode and did not depend on the external mechanisms of signal transduction mediated through membrane receptors.

Activity of caspase-3 in the RV myocardium also increased in focal LV ischemia at all three terms of the experiment (Table 2). Similarly as in LV, activity of caspase-8 on day 3 virtually did not differ from the control, which also indicated realization of apoptosis mechanisms by only the internal signal route.

The data characterizing myocardial contractility in LV ischemia are presented in Table 3.

Left ventricular RIP decreased significantly on day 1 of the experiment. Later a trend to gradual normalization of the parameter was observed, but it remained significantly below the control up to day 5. The time course of LV MIP was similar. These data indicated justified reduction of LV myocardial contractility during the acute period of its ischemia.

In RV, both RIP and MIP sharply increased by the end of day 1. Later these parameters gradually decreased. These changes seemed to be due to a significant increase of hemodynamic load of RV under conditions of reduced LV contractility.

In order to clear out possible mechanisms of the relationship between CMC apoptosis induction and changes in intracardiac hemodynamics in focal ischemia, we carried out analysis of correlations of the LV and RV means.

The data indicate a strong negative correlation between LV RIP and caspase-3 activity (-0.856) and between LV MIP and caspase-3 activity in LV (-0.857). Positive correlations between RV RIP and caspase-3 activity and between RV MIP and caspase-3 activity were found (0.886 and 0.647, respectively). Two mechanisms underlying this relationship are probable: LV contractility drops as a result of CMC apoptosis effected by caspase-3 or the caspase cascade is induced by reduction of LV myocardial contractility. However the direct relationship between the phenomena observed in this study has been doubted. Judging from the degree of caspase-3 activity elevation in LV, intensification of CMC apoptosis was not so sharp as to cause so great a reduction of myocardial contractility. However, the possibility of CMC apoptosis induction as a result of myocardial contractility reduction has not been proven. Another interpretation of the results of analysis of correlations for LV seems more probable. It seems that the reduction of LV contractility and CMC apoptosis were caused by the same factor, most likely hypoxia in ischemic CMC. It is known that intense lasting oxygen deficiency in cells stimulates the mitochondrial mechanisms of their apoptosis [11]. For example, it was found that hypoxic exposure leads to induction in the cells of factor 1α, which stabilizes p53 proapoptotic protein [9] and stimulates the production of BNIP3 and NIX proteins binding, thus inhibiting activity of Bcl-2 antiapoptotic protein [6,7,14]. In addition, hypoxia disordering electron and proton transport along the respiratory chain leads to reduction of the membrane potential and sharp elevation of the permeability of internal mitochondrial membrane. As a result, cytochrome C (a most important intracellular inductor of the caspase cascade [11]) is intensely released into the cytoplasm from the mitochondria.

TABLE 2. Activities of Caspase-3 and Caspase-8 in RV Myocardium in Focal LV Ischemia (*M*±*m*)

Group		Caspase-3	Caspase-8
Control		0.08±0.01	0.28±0.06
Experimental	day 1	0.14±0.02*	Not measured
	day 3	0.17±0.03*	0.34±0.04
	day 5	0.16±0.02*	Not measured

TABLE 3. Cardiac Contractility in Focal LV Ischemia (*M*±*m*; mm Hg)

Group		LV		RV	
		RIP	MIP	RIP	MIP
Control		135.7±3.2	182.8±3.4	31.48±0.55	47.0±0.95
Experimental	day 1	110.6±3.1*	131.8±3.7*	50.12±1.0*	59.8±1.53*
	day 3	115.6±2.8*	139.9±3.8*	47.1±1.2*	53.3±1.59*
	day 5	117.4±1.1*	144.6±3.8*	46.6±0.81*	55.6±1.33*

The RV is characterized by a strong positive correlation between RV RIP and caspase-3 activity and medium positive correlation between RV MIP and caspase-3 activity, which is justified and indicates induction of apoptosis enzymatic mechanisms as a result of hemodynamic overload of RV. This is in good agreement with data of other authors indicating that CMC apoptosis increases in response to myocardial overload [2,8,12].

The results led us to the following conclusions. First, LV myocardium under conditions of its focal ischemia is characterized by elevated caspase-3 activity and reduced contractility. A strong negative correlation between caspase-3 activity and parameters of LV function indirectly indicates potentiation of CMC apoptosis mechanisms by the internal (mitochondrial) route under the effect of hypoxia. Second, the caspase cascade is also stimulated in RV as well, most probably due to RV hemodynamic overload because of deterioration of LV contractility. Third, activity of caspase-8 virtually does not change in both cardiac ventricles myocardium, which suggests that external (receptor-mediated) mechanisms are not involved in CMC apoptosis induction in LV ischemia and RV overload. Hence, the apoptotic signal is transmitted in both cardiac ventricles only via the internal (mitochondrial) route.

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