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Induction of Caspase Cascade as a Nonspecific Response to Myocardial Damage

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In three experimental series, acute hemodynamic overload of the left ventricle, focal ischemia of the left ventricle, and diphtheritic intoxication were modeled in rabbits. On days 1, 3, and 5 of the experiments, activity of myocardial caspase-3 and caspase-8 were measured separately in the left and right ventricles. In the left ventricle, caspase-3 activity increased in all 3 modeled pathological processes, while in the right ventricle this parameter increased during acute overload and ischemic injury to the left ventricle. Caspase-8 activity increased only in the left ventricle during its hemodynamic overload and remained unchanged in other cases. It was concluded that induction of the caspase cascade can be considered as a nonspecific response to myocardial damage. In this case, specific mechanisms responsible for generation and transmission of apoptotic stimuli in cardiomyocytes have unique features.

Key Words: *caspase; myocardium; ischemia; congestion; diphtheria.*

Various aspects of cardiomyocyte (CMC) apoptosis in various diseases of the cardiovascular system are intensively discussed over the last decade. It was shown that apoptotic activity in the myocardium increases in arterial hypertension [5], progressive dilated cardiomyopathy [1], and myocarditis [4,6]. There is no consensus on the role of programmed cell death in changes of tissue homeostasis in ischemic myocardium. Some researchers report that myocardial ischemia is accompanied by increased apoptosis of CMC [2,3], but others hold the opposite point of view [9]. It is noteworthy that some time ago, the notion that cardiac myocyte apoptosis is a factor contributing to the transition from primary pathology of the heart to the formation of chronic heart failure became widespread [7,8]. Taking into account the leading role of cysteine proteases (caspases) in apoptotic cell death, it can be hypothesized that induction of caspase cascade is a nonspecific response to myocardial damage. For verification of this hypothesis we measured activities of effector caspase-3 and initiator caspase-8 in three fundamentally different models of heart pathology.

MATERIALS AND METHODS

Three series of experiments were conducted on male chinchilla rabbits weighing 3.0-3.5 kg. In each series, 20 animals were used; they were divided into 4 groups: control group (intact rabbits), and 3 experimental groups (days 1, 3, and 5 after the start of the pathological process). In series I, acute hemodynamic overload of the left ventricle (LV) was created by narrowing the ascending aorta by $^1/_3$ with a metal spiral. In series II, focal ischemia of LV was induced by ligation of the descending branch of the left coronary artery at the boundary between its middle and lower

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thirds. In series III, diphtheritic intoxication was modeled by a single intravenous injection of native diphtheria toxin in a dose of 0.3 minimal lethal dose per 1 kg body weight (previously titrated on guinea pigs). All animal studies were conducted in accordance to the rules of animal experiments (Order of USSR Ministry of Health, No. 755, 12.08.1977).

In all animals, the chest was opened and extirpation of the heart performed under general anesthesia. Myocardial specimens weighing 200-250 mg were cut from the wall of LV and RV. In animals with acute focal myocardial ischemia, LV specimens were taken from areas of viable myocardium adjacent to the zone of necrosis. Myocardial specimens (LV and RV separately) were minced in a WiseTis homogenizer (series HG-15) with 8 mm rotor at 4500 rpm. The isolation medium contained 20 mM HEPES (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol, and a cocktail of protease inhibitors (104 mM AEBSF, 0.08 mM aprotinin, 1.5 mM pepstatin A, 2 mM leupeptin, 4 mM bestatin, 1.4 mM E-64) added at a ratio of 100:1 (all reagents from Sigma). The homogenates were centrifuged in a Heraeus fresco 17 microfuge (Thermo Electron LED GMBH; hereinafter the equipment of the Center for Collective Use of Peoples' Friendship University of Russia) at 15,000*g* and 4°C for 30 min, and the supernatants were used for measuring activities of caspase-3 and caspase-8.

Activity of caspase-3 was determined 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 microplate for 95 min at 37°C in a reaction buffer (20 mM HEPES pH 7.4, 2 mM EDTA, 5 mM dithiothreitol, and 0.1% CHAPS) in two parallel samples, one of which contained 20 nM Ac-DEVD-pNA and another contained 20 nM Ac-DEVD-pNA and 2 nM Ac-DEVD-CHO, a specific inhibitor of caspase-3. Optical density was recorded every 10 min by ELISA-reader Sunrise (Tecan) at 405 nm. Activity of caspase-3 was calculated by the difference in the rate of the substrate cleavage in samples with and without inhibitor using calibration curve for standard pNA.

Activity of caspase-8 was estimated by the colorimetric method by the rate of cleavage of synthetic substrate Ac-IETD-pNA (N-acetyl-Ile-Glu-Thre-Asp-nitroaniline; Sigma). The supernatant was incubated in 96-well microtiter plates at 37°C in reaction buffer (20 mM HEPES pH 7.4, 2 mM EDTA, 5 mM dithiothreitol, 0.1% CHAPS, 5% sucrose) in two parallel samples, one of which contained 20 nM of Ac-IETD-pNA, and another contained 20 nM of Ac-IETD-pNA and 0.05 nM Ac-IETD-CHO, a specific caspase-8 inhibitor. Optical density was recorded every 10 min over 1 h by ELISA reader Sunrise (Tecan) at 405 nm. Activity of caspase-8 was calculated by the difference in the rate of the substrate cleavage in samples with and without inhibitor using calibration curve for standard pNA.

The data were treated statistically using software developed by the Department of General Pathology and Pathophysiology of People's Friendship University, as well as software Biostat. When analyzing the results Student's *t* test was used (differences between the means were significant at $p \leq 0.05$). The relationship between individual processes and phenomena was established by correlation analysis.

RESULTS

On day 1 after stenosis of the ascending aorta, a significant increase in specific activity of caspase-3 in the LV myocardium was observed (Fig. 1, *a*). No significant differences in this parameter from the control were found in RV. By day 3, caspase-3 activity remained unchanged in LV and increased and signifi-

TABLE. 1. Activity of Caspase-8 in Ventricular Myocardium in Different Types of Pathological Processes in the Heart (*M±m*)

Model of heart pathology		Control	Days of experiment		
			1	3	5
LV	LV overload	0.19±0.05	0.39±0.05	n.d.	n.d.
	Focal ischemia of LV	0.19±0.05	n.d.	0.27±0.04	n.d.
	Diphtheria intoxication	0.61±0.02	n.d.	n.d.	0.75±0.1
RV	LV overload	0.28±0.06	n.d.	0.35±0.03	n.d.
	Focal ischemia of LV	0.28±0.06	n.d.	0.34±0.04	n.d.
	Diphtheria intoxication	n.d.	n.d.	n.d.	n.d.

Note: * $p \leq 0.05$ compared to the control; n.d.: not determined.

cantly surpassed the control group in RV. On day 5, caspase-3 activity in the myocardium of both ventricles tended to decrease; in LV this parameter did not differ significantly from the control, while in RV it still significantly exceeded the control values.

In focal ischemic damage to LV, on day 1 day of the experiment of caspase-3 activity increased both in the macroscopically intact area of the LV and in RV myocardium (Fig.1, *b*). By the end of day 3, this parameter continued to increase and reaches the peak level in both ventricles. By day 5, activity of caspase-3 in LV significantly decreased and returned to control values, while in RV it did not change compared to the previous term.

Dynamics of caspase-3 activity in the myocardium of LV and RV during diphtheria intoxication is shown in Figure 1, *c*.

In LV, these parameters remained unchanged on day 1, tended to increase on day 3, and on day 5 the differences from the control became significant. In RV, caspase-3 activity did not differ significantly from the normal at all terms of the study. It can be assumed the diphtheria toxin in RV reduces activity of caspase-3on the one hand and induces certain metabolic changes activating caspase cascade, on the other. The overall effect consists in the absence of any dynamics. In more massive LV, the amount of injected toxin is apparently insufficient for direct suppression of caspase-3, but metabolic abnormalities are comparable to the severity of these in RV, which leads to activation of caspase mechanisms.

Taking into account the fact that caspase-3 activity significantly increased in all the three models of myocardial damage based on fundamentally different pathogenetic mechanisms, we believe that induction of caspase cascade can be considered as a nonspecific response to myocardial damage.

Activity of caspase-8 in ventricular myocardium was studied (Table 1). In all series, measurements were performed only in the control groups and when activity of caspase-3 reached the maximum value, because caspase-8 is an initiator enzyme and its activity reflects only the involvement of the mechanisms of external signaling in the process of apoptosis stimulation.

The findings suggest that the studied parameter significantly surpassed the normal only in LV myocardium during its acute hemodynamic overload. In other cases the differences were insignificant. Hence, the caspase cascade is activated in LV upon stenosis of the ascending aorta only via the external (receptor-mediated), or by external and internal (mitochondrial) pathways. During ischemic injury of LV, myocardial damage induced by diphtheria toxin, and RV overload caused by pulmonary congestion, activation of caspases occurs only by the mitochondrial pathway. Thus,

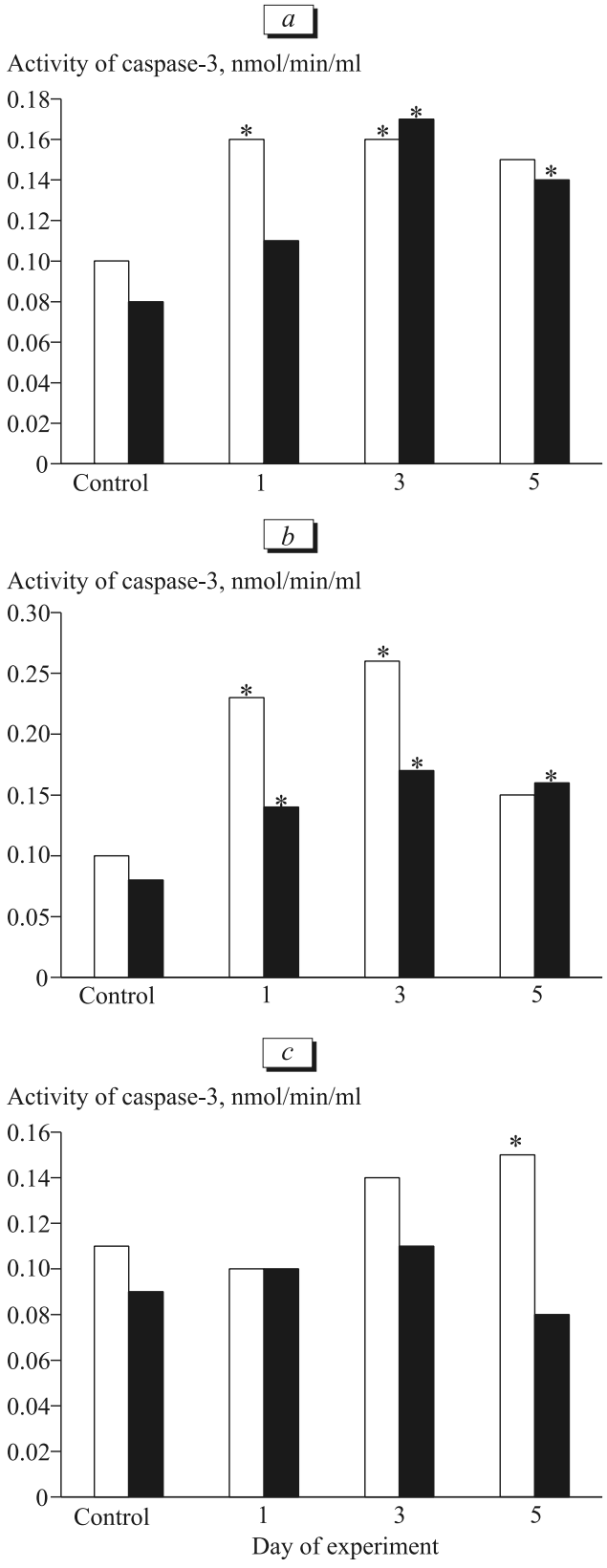

Fig. 1. Activity of caspase-3 in the myocardium of the heart ventricles of rabbits in acute hemodynamic overload of the left ventricle (*a*), focal ischemia of the left ventricle (*b*) and diphtheria intoxication (*c*). Light bars: LV, dark bars: RV. *$p \leq 0.05$ compared to the control.

despite the nonspecific nature of the caspase cascade response to heart damage, the specific mechanisms responsible for the induction and transmission of the apoptotic stimuli into cardiomyocytes have unique features.

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