ISSN 1990-7508, Biochemistry (Moscow) Supplement Series B: Biomedical Chemistry, 2011, Vol. 5, No. 2, pp. 171–174. © Pleiades Publishing, Ltd., 2011.
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Assessment of Caspase 3 Activity in Rabbit Myocardial Tissue during Experimental Hemodynamic Overload of the Left Ventricle of the Heart

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Received February 26, 2010

Abstract—It is well known that chronic overload of the cardiac left ventricle is accompanied by an increase in the cardiomyocyte apoptosis rate. However, direction and extent of changes in programmed cell death under an acute overload of the left ventricle still requires detailed investigation (as its pathogenesis significantly differs from chronic overload). Caspase-3 activity has been investigated in left ventricle myocardium of rabbits on days 1, 3, and 5 after modeling of left ventricle hemodynamic overload caused by experimental stenosis of the ascending aorta. Control group included intact animals. It was found that caspase-3 activity significantly increased in both ventricles on day 1; it increased more than twofold above control values on day 3 and decreased up to nearly control values on day 5. Based on these data it was concluded that the acute hemodynamic overload of the left ventricle may be a cause of increased apoptosis in the myocardial tissue of both cardiac ventricles during first days of the pathological process.

Key words: myocardium, left ventricle, right ventricle, overload, caspase, apoptosis.

DOI: 10.1134/S1990750811020028

INTRODUCTION

Aspartate-specific cysteine proteases that belong to the caspase ICE/CED-3 family (ICE/CED for 3 – interleukin-1 beta-converting enzyme) play a key role in the realization of cell death via the apoptotic mechanism in most mammalian cells and therefore they are considered as a marker of apoptosis [1, 2]. More than 15 members of this family are known to date; these enzymes are subdivided into three subgroups: initiator, anti-inflammatory and effector caspases. Effector caspases form so-called caspase cascade responsible for induction, transduction, and amplification of intracellular apoptotic signals [3].

Apoptosis may be triggered by both signals from external stimuli transduced into cells via membrane death receptors (Fas-L and other ligands referred to the superfamily of tumor necrosis factor (TNF) receptors) [4, 5] and also by specific changes of intracellular molecules such as impairments on native DNA structure, which trigger the mitochondrial mechanism of programmed cell death [6]. Realization of the first of these mechanisms involves caspase 8 [7, 8]. The second mechanism is associated with release of mitochondrial cytochrome *c* and other factors activating the caspase cascade via the mitochondrial permeability transition pore into cytoplasm [9, 10]. However, in any variant caspase 3 is a final enzyme of the caspase chain required for chromatic condensation, DNA fragmentation followed by formation of apoptotic bodies [11]. Consequently, this enzyme may be used as a universal marker for assessment of programmed cell death regardless the pathways of its realization. High specificity of caspase 3 for apoptotic process is another advantage of this method of apoptosis evaluation.

During the last decade there were several studies on cardiomyocyte apoptosis in various forms of cardiovascular pathology; these studies employed determination of myocardial caspase 3. Some experimental and clinical data suggest that chronic overload of left ventricle induced by arterial hypertension and slow progressing stenosis of the thoracic aorta is accompanied by a sharp increase of myocardial caspase 3 [12-14].

However, extent of apoptosis under an acute hemodynamic overload of the left ventricle, still requires detailed investigation as its pathogenesis significantly differs from the chronic overload. Thu, in this study we have investigated activity of caspase 3 in the rabbit myocardial tissue during acute hemodynamic overload of the left ventricle.

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Table 1. Left ventricle myocardial DEVD cleaving activity (pmol AMC/min/mg of protein) in the rabbit heart under experimental stenosis of the ascending aorta

Parameter	Control	Time course of the process		
		Day 1	Day 3	Day 5
S	0.70 ± 0.06	1.31 ± 0.21*	1.55 ± 0.29*	1.19 ± 0.21*
S + I	0.38 ± 0.04	0.41 ± 0.08	0.51 ± 0.04*	0.54 ± 0.05*
S – SI	0.32 ± 0.08	0.90 ± 0.26*	1.04 ± 0.26*	0.64 ± 0.19

Note: Here and in Table 2 S—activity of proteolytic enzymes assayed with the caspase 3 substrate, Ac-DEVD-AMC (total DEVD cleaving activity), S + I—activity assayed with the substrate and in the presence of a specific caspase 3 inhibitor, Ac-DEVD-CHO (nonspecific remaining proteolytic activity), S-SI—caspase 3 activity evaluated as the difference between activities in S and S + I. Asterisk shows statistically significant difference from the control level, $p \le 0.05$. Data represent mean ± SEM of 5 experiments.

MATERIALS AND METHODS

Twenty Shinshilla rabbits (3.0–3.5 kg) were used in experiments. Animals were subdivided into four groups (in each group $n = 5$): one control group (intact rabbits) and thee experimental groups analyzed on day 1, day 3, and day 5, respectively. For modeling of acute hemodynamic overload rabbits of all experimental groups underwent a surgical operation under general anesthesia. This operation included stenosis of the ascending aorta (by 1/3) by screwing a metal coil of a smaller diameter.

In accordance with subdivision into experimental groups hearts were extirpated from rabbits under general anesthesia for subsequent biochemical studies.

The myocardial tissue of left and right ventricles were cut separately in a special mechanic device and then homogenized in the isolation medium containing 20 mM Hepes, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 1 mM phenylmethylsulfonylfluoride, 10 μg/ml aprotinin, 10 μg/ml pepstatin A, and 10 μg/ml leupeptin, pH 7.5 (Sigma, USA) using a Potter S homogenizer (Teflon—glass, Braun, Germany) at the rate 1500 rpm. Homogenates were centrifuged at 15000 *g* for 30 min at 4°C and resultant supernatants were used for assessment of caspase 3 activity.

Caspase 3 activity was assayed by a fluorometric method by the rate of cleavage of the synthetic fluorogenic substrate Ac-DEVD-AMC (N-acetyl-Asp-Glu-Val-Asp-7-amino-4-(trifluoromethyl)coumarin; Biomol, USA) [15]. The supernatant was incubated for 60 min at 37°C in the reaction buffer (50 mM Hepes, pH 7.5, 10% sucrose, 10 mM DTT, 0.1% CHAPS) in two parallel samples, one of which contained 100 μM Ac-DEVD-AMC, whereas the other one contained 100 μM Ac-DEVD-AMC and 10 μM Ac-DEVD-CHO, a specific inhibitor of caspase 3. Fluorescence was registered every 10 min using a Hitachi F-3000 spectrofluorimeter (excitation and emission wavelengths of 380 and 440 nm, respectively). Caspase 3 activity was calculated as the difference between reaction rate of substrate cleavage in the samples lacking and containing caspase inhibitor using a calibration curve of fluorescence of an AMC standard. Protein concentration in supernatants was determined by the method of Bradford [16].

All results were treated statistically using programs developed at the Department of General Pathology and Pathological Physiology (Russian University of Peoples' Friendship), the Biostat program, and Student's t test. Mean, the standard error of the mean and standard deviation were calculated. Statistical differences between mean values were considered as statistically significant at $p \le 0.05$.

RESULTS AND DISCUSSION

Table 1 shows that the total Ac-DEVD cleaving activity in the left ventricular myocardium significantly increased by the end of day 1 of its hemodynamic overload. After three days this parameter demonstrated further increase: it was 2-fold higher than the control value. On day 5 the total Ac-DEVD cleaving activity demonstrated some decrease compared with previous postoperative time interval, but nevertheless it exceeded control level.

In samples containing both substrate and inhibitor of caspase 3, remaining catalytic activity attributed to other proteolytic enzymes also gradually increased reaching the level of statistically significant differences at day 3 and day 5. However, this increase was less pronounced than the increase in the total Ac-DEVD cleaving activity (assayed without the caspase 3 inhibitor) (Fig. 1).

This increase in activity of enzymes cleaving the substrate of caspase 3 in the presence of its inhibitor may be explained by postoperative activation of other proteolytic enzymes that may also contribute to the cleavage of the caspase 3 substrate.

Specific activity of caspase 3 evaluated by the difference in the Ac-DEVD cleaving activity assayed without and the caspase 3 inhibitor significantly increased on day 1 and demonstrated further increase on day 3. However, on day 5 it decreased compared with previous postoperative intervals and insignificantly exceeded control values.

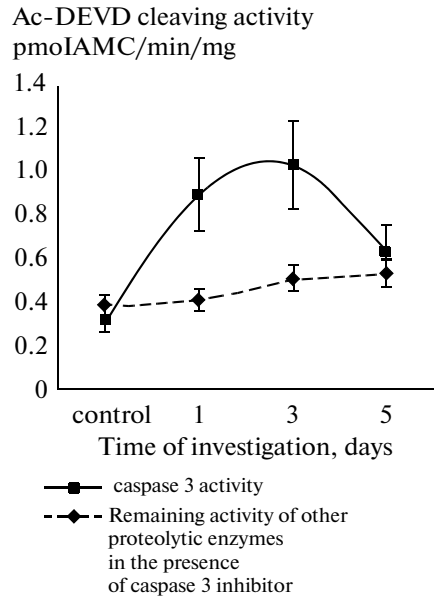

Fig. 1. Time course of Ac-DEVD cleaving activity in the left ventricle myocardium of the rabbit heart during its acute hemodynamic overload.

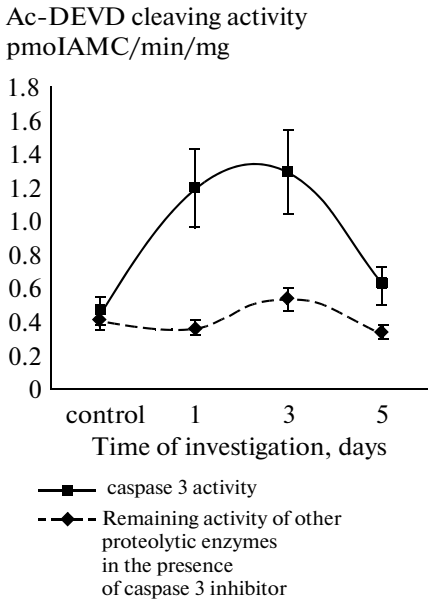

Fig. 2. Time course of Ac-DEVD cleaving activity in the right ventricle myocardium of the rabbit heart during acute hemodynamic overload of the left ventricle.

In the right ventricular myocardium caspase 3 activity significantly increased on day 1 after acute left ventricular overload and reached maximum on day 3; however on day 5 its activity sharply decreased up to the control level. This suggests inactivation of this enzyme that performed its proapoptotic functions at the end of experiment.

In the presence of the caspase 3 inhibitor, the Ac-DEVD cleaving activity significantly increased only on day 3 and this increase was smaller than in the samples without this inhibitor. This suggests that activity of other proteases that contribute to cleavage of the caspase 3 substrate insignificantly changed compared with control.

As in the case of left ventricle, differences in the Ac-DEVD cleaving activity assayed without and with the caspase 3 inhibitor were statistically significant over all postoperative periods studied (Fig. 2).

Thus, caspase 3 activity demonstrated similar changes in myocardium of left and right ventricles: three first days of the pathological process were characterized by significant increase with maximum observed on day 3, on day 5 this parameter decreased and this decrease was more evident in the right ventricular myocardium where caspase 3 activity basically return to the control level. However, in the left ventricular myocardium the increase in caspase 3 activity was somewhat higher than in the right ventricular myocardium.

Results of this study give some explanations to events observed in myocardium under conditions of its acute overload. First, early stages of cardiac output adaptation to increased resistance are characterized by a sharp increase of myocardial caspase 3 in left and right ventricles; this may suggest intensification of apoptosis in myocardial cells, presumably cardiomyocytes. Since mammalian cardiomyocytes exhibit limited regeneration capacity [17] their death via apoptosis or necrosis inevitably results in decreased myocardial contractile capacity (or at least in deficit of its reserve capacities). This suggestion is consistent with results of studies that elucidated correlation between cardiomyocyte apoptosis and the development of cardiac failure [17-19].

The other important but less expected result of this study consists in the increase of right ventricular caspase 3 activity during overload of the left ventricle; this also suggests augmentation of apoptosis. The mechanism of this increase may be also associated with hemodynamic overload of the right ventricle, which may be attributed to congestion in the pulmonary circulation, possibly developed due to the increase in left ventricular end-diastolic volume. However, in the case of the right ventricle, the loss of cardiomyocytes is more critical as it is less adapted to overload and is "a weak link" in the heart [20], responsible for its rapid decompensation.

Thus, one may suggest that increased apoptosis of cardiomyocytes represents a cardiac response not only to chronic but also acute ventricular overload. How this could be explained? In our previous studies we found that early stages of experimental arterial hypertension are accompanied by development of two parallel processes in the myocardium: myofibrillar hypertrophy and augmentation of apoptosis in cardiomyocytes and there was potent significant correlation

between these processes. Based on these results we concluded that apoptosis is a factor limiting myocardial hypertrophy during gradually increasing hemodynamic overload [21]. It is possible that augmentation of apoptosis in cardiomyocytes under acute overload also represents a preventive response to inevitable hypertrophy.

However, using results of our study and literature data it is hard to propose mechanisms responsible for augmentation of myocardial apoptosis during excessive overload of the left ventricle. It is known that during arterial hypertension augmentation of programmed cell death of cardiomyocytes is associated with increased content of blood angiotensin II [22]. However, experiments on rabbits with chronic cardiac overload induced by metal coil screwed on the ascending aorta within several days after birth revealed augmentation of apoptosis in cardiomyocytes [12]. This augmentation may be attributed increased resistance to cardiac output rather than to the effect of plasma angiotensin II. It is possible that programmed cell death of cardiomyocytes during increased mechanical load is induced by increased degree of ventricular muscle stretch, which is accompanied by significant activation of Ca^{2+} entry into cells followed by calcium-dependent activation of the caspase cascade [23].

CONCLUSIONS

Acute experimental stenosis of the ascending aorta is accompanied by a sharp increase of caspase 3 activity in the myocardium of both ventricles. This suggests that acute hemodynamic overload of left ventricle is a cause for increased apoptosis in the myocardium of both cardiac ventricles during first several days after the onset of the pathological process.

ACKNOWLEDGMENTS

This work was supported by the Federal Targeted Program "Scientific and Scientific-Pedagogical Personnel of the Innovative Russia" in 2009—2013.

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