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Ultrastructural changes associated with myocardial apoptosis, in failing rat hearts induced by volume overload☆

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ABSTRACT

Background: Myocardial apoptosis has been discussed to play a pivotal role in the development and progression of congestive heart failure (CHF). However, recently there is doubt on the evidence of myocardial apoptosis in heart failure as information on ultrastructural changes by electron microscopy is still scarce. This project therefore aimed to detect direct morphological evidence of myocardial apoptosis in an experimental heart failure model.
Method: Following IRB approval, an aortocaval fistula (ACF) was induced in male Wistar rats using a 16G needle. 28 ± 2 days following ACF rats were examined by hemodynamic measurements, Western blot, immunofluorescence confocal and electron microscopic analysis.
Results: Within 28 ± 2 days of ACF heart (3.8 ± 0.1 vs. 6.6 ± 0.3 mg/g) and lung (3.7 ± 0.2 vs. 6.9 ± 0.5 mg/g) weight indices significantly increased in the ACF group accompanied by a restriction in systolic (LVEF: 72 ± 2 vs. 39 ± 3%) and diastolic ($dP/dt_{min.}$: −10,435 ± 942 vs. −5982 ± 745 mmHg/s) function ($p < 0.01$). Activated caspase-3 was significantly increased in failing hearts concomitant with mitochondrial leakage of cytochrome c into the cytosol. Finally, electron microscopy of the left ventricle (LV) of ACF rats revealed pronounced ultrastructural changes in >70% of examined cardiomyocytes, such as nuclear chromatin condensation, myofibril loss and disarray, contour irregularities and amorphous dense bodies, mitochondriosis and damaged cell–cell-contacts between cardiomyocytes.
Conclusions: Volume overload induced heart failure is associated with activation of the mitochondrial apoptotic pathway. In addition, electron microscopy of the LV revealed direct ultrastructural evidence of extended myocardial apoptosis in ACF rats.

1. Introduction

Congestive heart failure (CHF) is defined as a pathophysiologic state in which an abnormality of cardiac structure and function resulting in the heart's inability to deliver the appropriate amount of oxygen to cover tissues requirements [1]. Despite improvements in CHF treatment, CHF is still associated with a high mortality and its prevalence is rising. Among others, coronary artery disease is the leading cause of CHF and several CHF models have been established in experimental settings [2]. Pathological remodeling of the heart with dilation and impaired contractility present maladaptive changes in cardiomyocytes and extracellular matrix.

Interestingly, progressive deterioration of left ventricular function occurs despite the absence of clinically apparent intercurrent adverse events [3]. In this context, cardiomyocyte apoptosis during left ventricular remodeling and cardiac dysfunction has been suggested to contribute to the progression of heart failure [4,5]. Since 1996, apoptosis occurring in cells with activated apoptotic signaling mechanisms has been recognized as one form of cardiomyocyte cell death in rats due to ischemia [6]. Then, involvement of myocardial apoptosis in the progression of heart failure became evident [5,7–9] independent of the cause of heart failure, e.g. myocardial infarction [10], pressure [11–13] and volume overload [14–16]. Especially volume overload seems to be susceptible to apoptosis detected by means of Western blot and light microscopy [17]. Interestingly, activation of apoptotic pathways may contribute to cardiac dysfunction in heart failure independent of cell loss ("apoptosis interruptus") [18–21].

Morphological changes related to apoptosis have mostly been defined by light microscopy showing cell pyknosis, volume reduction and plasma membrane blebbing [22]. However, recently there is doubt on the evidence of myocardial apoptosis in heart failure in animals and humans as information on ultrastructural changes by electron microscopy is still scarce [22]. This project therefore aimed to detect

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direct morphological evidence of myocardial apoptosis on the ultrastructural level in an experimental model of heart failure induced by a modified aortocaval fistula in rats.

2. Methods

Male Wistar rats, 280–300 g (Harlan Winkelmann, Borchen, Germany), were maintained at normal rat chow and water ad libitum. The animals were kept on a 12-h light–dark cycle. The experiments were approved by the local animal care authorities (Landesamt für Gesundheit und Soziales, Berlin, Germany) and were performed according to "The Guiding Principles for the Care and Use of Animals in Research and Teaching" adopted by the American Physiological Society (revised July 2010).

2.1. Experimental heart failure

Heart failure was induced by an infrarenal, aortocaval fistula (ACF) as previously described (n = 6) [23]. Briefly, a laparotomy was performed under Isoflurane anesthesia. While temporarily clamping the suprarenal aorta and manually compressing the venous vessels above and below the puncture site, the abdominal aorta was punctured with a 16G disposable needle distal to the renal arteries. Then the needle was advanced into the adjacent inferior vena cava and withdrawn. The aortic puncture site was sealed with a drop of cyanoacrylate glue. Sham-operated control animals were treated identically, except that no puncture of the vessels was performed (n = 6).

2.2. Hemodynamic measurements

28 ± 2 days after fistula induction hemodynamic measurements using the "closed chest" method in spontaneously breathing rats were performed under tiletamine/zolazepam anesthesia (Zoletil®, 10 mg/kg s.c. followed by 50 mg/kg i.m.) as described earlier [23,24]. All measurements were registered and analyzed by the PowerLab®-system/-software (AD Instruments, New Zealand). After tracheotomy a PE-50 tubing catheter was inserted via the left jugular vein into the superior vena cava for assessment of central venous pressure. Arterial blood pressure was measured by cannulating the right carotid artery with a micro-tip pressure-volume conductance catheter (Millar®, SPR- 838 NR). Intraventricular pressures and volumes were registered by further advancing the catheter into the left ventricle and optimizing its position aiming for maximal stroke volume (SV). For measurement of the parallel conductance volume 100 μl of 15% saline was injected into the central venous line as a correction factor for the blood–left ventricle (LV) tissue interface. Heart rate was derived from the ECG signal. All measurements were registered and analyzed by the PowerLab®-system/-software (AD Instruments, New Zealand).

2.3. Activated caspase-3 Western blot

To determine the protein level of activated caspase-3 myocardial tissue of the whole heart of control and ACF rats was solubilized according to Weems and colleagues [53] and Western blot analysis was performed as previously described by using a specific antiserum for activated caspase-3 [25]. The samples were homogenized in boiling SDS sample buffer (100 mM Tris, 2% SDS, 20% glycerol). The protein concentration was measured using a BCA assay (Pierce). 2-Mercaptoethanol and bromophenol blue was added before loading. The extracts were separated by SDS-PAGE (12%) using 60 μg protein per lane and then transferred onto nitrocellulose filters. The filters were blocked in 5% milk for 90 min and incubated with the following antibody: cleaved caspase-3 (Santa Cruz, No. 9664, 1:500, in 5% milk) overnight at 4 °C. After incubation with the secondary antibody (per-oxidase-conjugated anti-rabbit, 1:10.000, Jackson ImmunoResearch, No. 111-035-144) for 2 h at room temperature, reactive bands were digitally visualized in ECL solutions (SuperSignal West Pico, Thermo Scientific) in ChemiDoc MP Imager. Western blots were performed as three independent experiments in duplicate. The integrated optical density (OID) was calculated by use of Java Image processing and analysis software (Image J, NIH) as described previously [26].

2.4. Immunofluorescence and electron microscopy

For electron microscopy and double immunofluorescence staining of the LV rats (control: n = 3; ACF: n = 3) were deeply anesthetized with tiletamine/zolazepam (Zoletil®) and transcardially perfused with 100 ml warm saline, followed by 300 ml 4% (w/v) paraformaldehyde in 0.16 M phosphate buffer solution (pH 7.4). After perfusion the hearts were removed and then cryoprotected overnight at 4 °C in PBS containing 10% sucrose. For immunofluorescence evaluation, the tissues were then embedded in tissue-Tek compound (OCT, Miles Inc. Elkhart, IN) and frozen. The tissues were cut into 50-μm-thick sections in a cryostat and the sections were collected in PBS (floating sections). For electron microscopy evaluation, samples were fixed in Karnovsky's fixative and then post-fixed in 2% OsO_4/0.1 M phosphate buffer. After rinsing and dehydration in ethanol, the samples were embedded in Epon (Plano, Marburg, FRG), ultrathin cuts made on a Reichert-Ultracut E, and contrasted with 2% uranyl acetate and lead citrate. A transmission electron microscope (Zeiss TEM10, Jena, Germany) was used to examine the samples. To quantify the ultrastructural findings, sections were examined for apoptotic and mitochondrial changes (MC) by counting 100 cells from 20 microscopic fields. The examination was performed in triplicate, and the results were provided as the mean values with S.D. from three independent experiments.

2.5. Immunohistochemistry of cytochrome c distribution

Double immunofluorescence staining of cytochrome c in the LV was performed as described previously [27,28]. The sections were incubated overnight with the following primary antibodies: cytochrome c 136 F3 (rabbit mAb, #4280, Cell Signalling®). After incubation with primary antibodies, the tissue sections were washed with PBS and then incubated with Texas Red conjugated goat anti-rabbit antibody (Vector Laboratories). Thereafter, sections were washed with PBS and the nuclei stained bright blue with 4′-6-Diamidino-2-phenylindole (DAPI) (0.1 μg/ml in PBS) (Sigma). Finally, the tissues were washed in PBS, mounted in vectashield (Vector Laboratories) and viewed under a Zeiss LSM 510 laser scanning microscope (Carl Zeiss, Göttingen, Germany).

2.6. Statistical analysis

All tests were performed using SPSS 20.0 software program. Results are expressed as mean ± SEM. Statistical significance among groups was analyzed by Students t-test when normal distribution was shown (Kolmogorov–Smirnov test). Otherwise, Mann Whitney U-test on ranks was used for two-group comparison. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Congestive heart failure

Values for body weight, heart and lung weight, and the organ weight/body weight ratio ("index") and hemodynamic parameters from the control and ACF group are given in Table 1. Heart and lung weight indices were significantly increased in the ACF group ($p < 0.01$). In addition, right (CVP) and left ventricular (LV-EDP) filling pressures were elevated and the maximum rate of pressure development and decay were significantly reduced ($p < 0.01$). Left ventricular end-diastolic volume (LVEDV) was significantly higher ($p < 0.01$) and left ventricular ejection fraction (LVEF) was significantly reduced ($p < 0.01$) in the ACF group.

3.2. Increase in activated caspase-3 protein

Immunoreactive bands representing activated caspase-3 specific proteins at the expected molecular weight of 19 kDa in the myocardium of ACF rats were identified by Western blot analysis (Fig. 1). Densitometric analysis revealed a nearly 2.5-fold increase in the OID of activated caspase-3 protein in the myocardium of ACF rats compared to controls ($p < 0.05$) (Fig. 1).

Table 1
Morphometric and hemodynamic data.

	Control (n = 6)	ACF (n = 6)
BW (g)	379 ± 16	372 ± 9
Heart (mg)	1447 ± 40	2438 ± 110*
Heart/BW (mg/g BW)	3.8 ± 0.1	6.6 ± 0.3*
Lung (mg)	1403 ± 30	2573 ± 180*
Lung/BW (mg/g BW)	3.7 ± 0.2	6.9 ± 0.5*
HR (min^{-1})	376 ± 13	333 ± 14
SBP (mmHg)	159 ± 6	122 ± 6*
DBP (mmHg)	121 ± 4	68 ± 2*
LVEDP (mmHg)	4.9 ± 0.3	13.7 ± 1.2*
CVP (mmHg)	0.3 ± 0.1	6.2 ± 0.8*
dP/dt max. (mmHg/s)	15,307 ± 1007	9547 ± 1382*
dP/dt min. (mmHg/s)	−10,435 ± 942	−5982 ± 745*
LVEDV (μl)	198 ± 6	646 ± 52*
SV (μl)	143 ± 6	251 ± 31*
LVEF (%)	72 ± 2	39 ± 3*

Values are means ± SEM; n = 6 rats/group. BW = body weight HR = heart rate; SBP = systolic blood pressure; DBP = diastolic blood pressure; LVEDP = left ventricular end-diastolic pressure; CVP = central venous pressure; dP/dt max. = maximum rate of pressure development of the left ventricle in systole; dP/dt min. = minimum rate of pressure decay of the left ventricle in diastole; LVEDV = left ventricular end-diastolic volume; SV = stroke volume; LVEF = left ventricular ejection fraction.

* Indicates a statistically significant difference between groups ($p < 0.01$).