

THE STUDY ON ATRIAL NATRIURETIC PEPTIDE OF CARDIOMYOCYTES IN A REMOTE POST-REPERFUSION PERIOD IN EXPERIMENT

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The aim of the investigation was to assess the intensity of rat atrial natriuretic peptide (ANP) accumulation and release in a remote post-reperfusion period after a 10-minute circulatory arrest using quantitative analysis of secretory cardiomyocyte granules, morphological and physiological methods.

Materials and Methods. The experiments were carried out on 19 non-linear male rats weighting 220–250 g. Total ischemia (10 min) was simulated by cardiovascular bundle compression according to V.G. Korpachev. The intensity of ANP accumulation and release processes was assessed by quantitative analysis of immunolabeled atrial myocyte granules in transmission electron microscope. We studied tissue rearrangement in myocardium at light-optical level. Physiological condition of animal was assessed in post-reperfusion period by heart rate variability and arterial pressure level.

Results. Rat atrial myocytes in a remote post-reperfusion period were found to have intensified ANP accumulation and release processes. An increased release proceeds against the background of synthetic and proliferative activity of fibroblasts and has a cardioprotective effect promoting the decrease of atherosclerosis development. Released ANP participates in cardiac rhythm recovery under elevated blood pressure.

Conclusion. Quantitative analysis of immunolabeled granules together with a complex of morphological and physiological methods indicates the intensification of atrial natriuretic peptide accumulation and release in rat atrial cardiomyocytes by the 60th day of post-reperfusion period.

Key words: atrial natriuretic peptide; ANP; remote post-reperfusion period.

Atrial natriuretic peptide (ANP) is the most active among numerous natriuretic peptides, which participate in the regulation of water-salt balance and hemodynamics due to arterial pressure (AP) reduction, and are antagonists of rennin-angiotensin-aldosterone system [1]. ANP was first found in atrial cardiomyocyte granules by De Bold et al. in 1981 but later was revealed in mast cells, in respiratory epithelium, pulmonary vein smooth muscle cells, hypothalamic neurons [2]. When ANP binds to A or B receptors on cellular membranes of target organs, membrane-bound guanylate cyclase transforms guanosine triphosphate in cyclic guanosine monophosphate (cGMP), which further activates protein kinases or phosphodiesterases and realizes ANP physiological effects. AP decreases due to the effect on kidneys by diuresis and natriuresis increase, and inhibition of rennin synthesis/secretion, as well as aldosterone in adrenals [1]. ANP promotes the decrease of fluid volume in blood flow due to vascular endothelial permeability increase and vasodilation

[3]. Apart from the above mentioned effects, ANP participates in lipid metabolism and inflammatory reactions. ANP concentration in blood is significantly increased in cardiac dysfunction and arterial hypertension, though cause-effect relations remain unclear due to insufficiency of data on ANP in health and disease [4]. Currently, there are no precise, sensitive and specific methods for ANP content determination due to its metabolic, structural and physiological characteristics; therefore, electron microscopy using immunocytochemistry can serve as a helpful tool for ANP study. The intensity of ANP synthesis, accumulation and excretion can be assessed using quantitative analysis of different types of secretory. Cardiomyocyte granules antigen-labeled to this peptide [5–7]. The technique is rather sensitive and informative, and combined with AP level and cardiac rhythm control enables to explain the processes proceeding in experimental animals.

The aim of the investigation was to assess the intensity of rat atrial natriuretic peptide accumulation and release

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in a remote post-reperfusion period after a 10-minute circulatory arrest using quantitative analysis of secretory cardiomyocyte granules, morphological and physiological methods.

Materials and Methods. The experiments were carried out on 19 non-linear male rats weighting 220–250 g. The study complies with laboratory routine guidance and European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (adopted in 18.03.1986 in Strasburg and approved in 15.06.2006 in Strasburg). Total ischemia was simulated by 10-minute cardiovascular bundle compression according to V.G. Korpachev [8].

Electron-microscopic analysis of the right atrial and left ventricular tissue samples of intact and experimental animals (60 days after post-reperfusion period — PRP) was carried out according to a standard technique [9]. Immunocytochemical reactions to reveal ANP localization were performed on ultrathin sections using polyclonal antibodies Rabbit anti-Atrial Natriuretic Factor (1-28) (rat) (Peninsula Laboratories, LLC, Bachem, USA) and antibodies Protein-A/Gold (15 nm) (EM Grade, Electron Microscopy Sciences, USA). The sections were counterstained by uranyl acetate, lead citrate and analyzed under transmission electron microscope Morgagni 268D (FEI, USA). According to one of the classifications used we distinguished two types of granules: A-type — “mature, storage”, and B-type — “deliquescent” [5–7]. A- and B-granules with peptide in atrial cardiomyocytes were calculated per field of vision (38×38 μm) [7].

LV tissue was studied at light optical level in intact rats and after 60 days of PRP. The samples were fixed in 10% formaldehyde solution and embedded in paraffin [9]. 5–7 μm sections prepared on microtome SM 2000R (Leica, Austria) were van Gieson’s stained (to reveal collagen fibers); and studied using light microscope Eclips 80i (Nikon, Japan) and program NIS-Elements BR 4.00.02. Percentage ratio of cardiomyocytes and collagen fibers per field of vision (2560×1920 μm) was determined. The findings were assessed using Mann–Whitney test.

We recorded initial ECG data and those obtained after 60 days of PRP in rats using “Polyspectrum-12” (“Neurosoft”, Russia). We analyzed heart rate variability (HRV) using the following parameters: mean RR-interval (R–R_m), standard deviation of R–R-intervals (SDNN), coefficient of variation (CV); spectral analysis parameters in normalized units (NU): total power of spectrum (TP), power of low-frequency and very-low-frequency (LF and VLF) and high-frequency (HF) spectrum. Nonlinear HRV parameters were assessed by method of R–R increment graphs plotting on phase plane (chaosgrams). A chaosgram was software-based divided into N₂, N₃, N₄₋₆ parameters, which reflected the number of waves with a definite number of points (2, 3, 4-6 respectively) [7, 10]. AP was measured through the carotid artery by invasive method using pressure probe MPX5050DP (Motorola, USA). The obtained signal was analyzed using software system “PowerGraph” V.2.0. The findings were statistically processed by program Statistica 10.0 using Friedman test and Wilcoxon test (p<0.05).

Results. On day 60 of PRP quantitative analysis of granules with ANP immunolabeled myocytes showed the increase of A-type granules by 60%, B-type – by 41%, total number of granules — by 53% compared to the values of intact animals (Fig. 1).

Light optical study of LV myocardium revealed marked interstitial edema and moderate cardiac muscle cells hypertrophy (Fig. 2, 3). Morphological analysis showed statistically significant increase of the area occupied by mature collagen fibers — by 3% compared to intact animals. Unstained area — with ground substance of connective tissue — amounted to 12% of total myocardial area. Cardiomyocyte area was reduced by 14% in comparison with intact values (Fig. 4).

Submicroscopic myocardial study of the right atrium and the left ventricle after 60 days of PRP revealed mosaic changes of cardiomyocytes: some cells had nuclei with marked karyolemma invaginations and chromatin aggregation. The nuclei in other cells had smooth contours, the nucleoli were determined, chromatin was homogeneously spread in karyoplasts. Cardiomyocyte

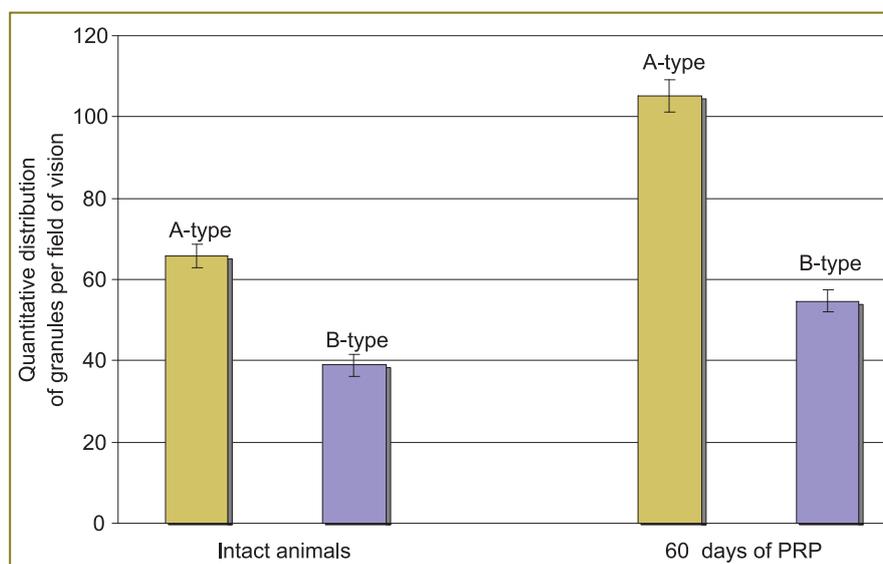


Fig. 1. Quantitative distribution of ANP granules after 60 days of post-reperfusion period (according to Mann–Whitney test)

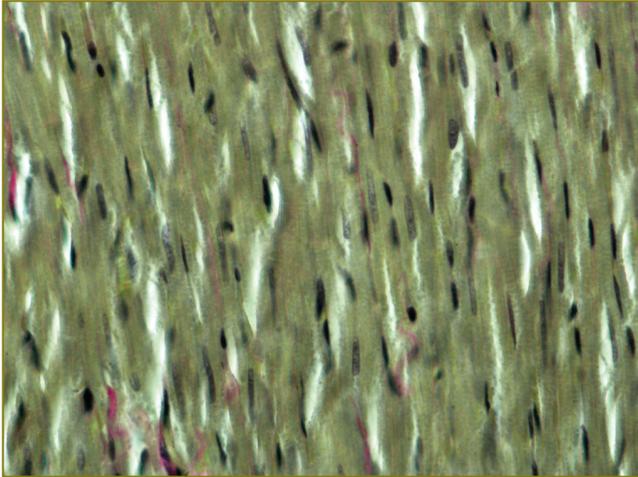


Fig. 2. Left ventricular myocardial structure of an intact rat. Ocular lens $\times 10$, object lens $\times 40$; van Gieson's stain

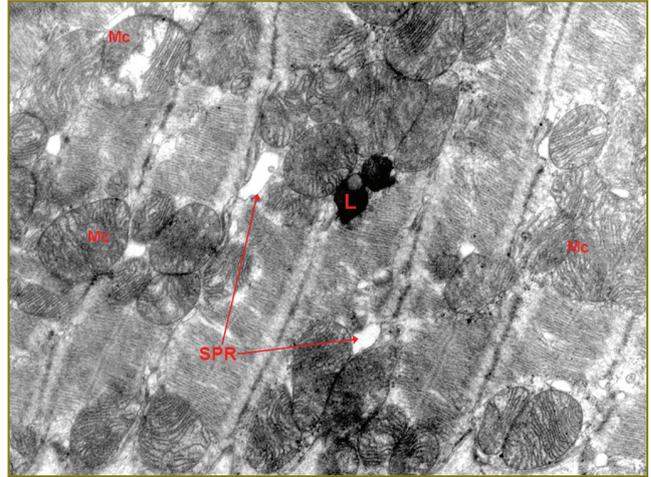


Fig. 5. Ultrastructure of rat left ventricular cardiomyocyte after 60 days of post-reperfusion period: Mc — mitochondria; L — secondary lysosomes; SPR — extended sarcoplasmic reticulum. $\times 8900$

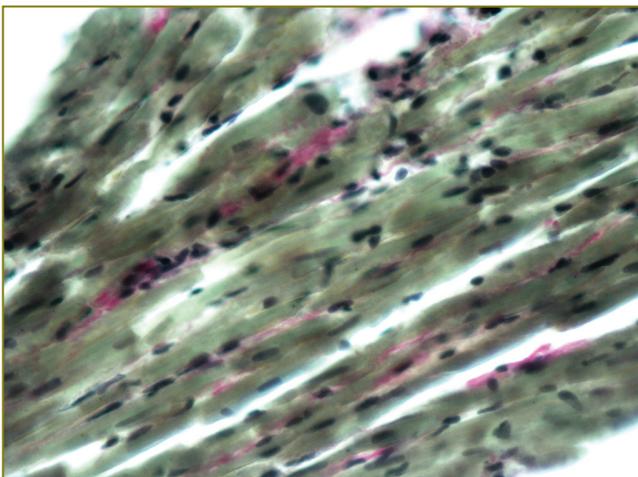


Fig. 3. Rat left ventricular myocardial structure after 60 days of post-reperfusion period. Ocular lens $\times 10$, object lens $\times 40$; van Gieson's stain

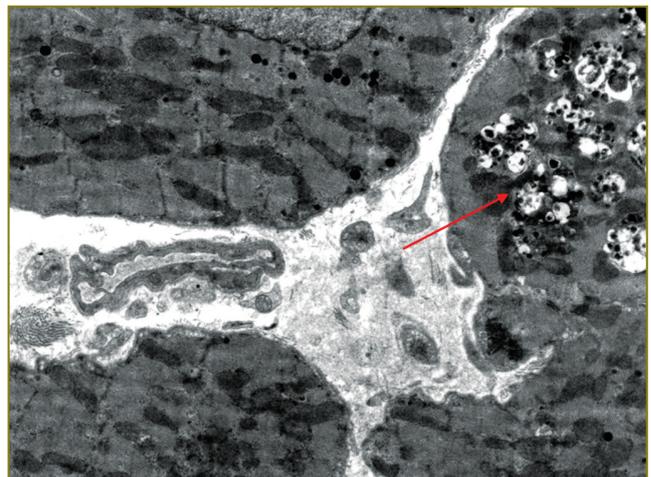


Fig. 6. Ultrastructure of rat right atrial myocardium after 60 days of post-reperfusion period; the arrow indicates cardiomyocyte in a state of apoptotic degeneration. $\times 4400$

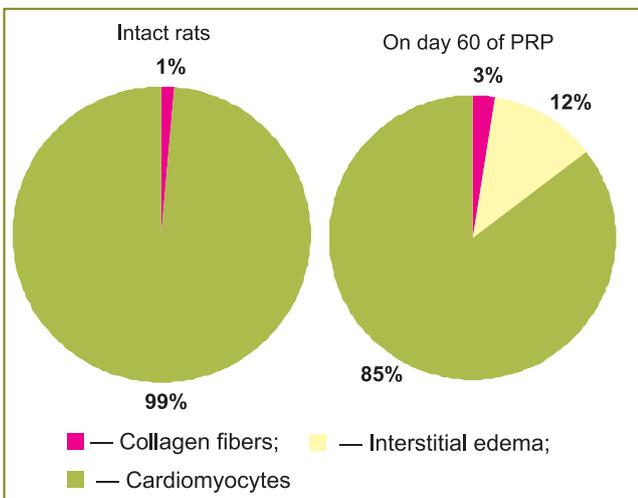


Fig. 4. The change in tissue component proportion in left ventricular myocardium in a remote post-reperfusion period

mitochondria mainly had condensed form; some organelles had matrix clarification and crista disorientation. Vacuole formation was found. In most cardiomyocyte myofibrils were clearly determined. Single cells had the areas with myofibril stratification. Significant dilatation of sarcoplasmic reticulum (SPR) in most cardiomyocytes was observed. There were found secondary lysosomes (Fig. 5). In the right atrial and left ventricular myocardium we revealed cardiomyocytes in a state of apoptotic degeneration (Fig. 6). In extracellular space we observed connective tissue overgrowth, in ground substance of which there was mature collagen and fibroblasts (Fig. 6).

By day 60 of post-reperfusion period HRV values did not statistically significantly differ from those of intact animals, except N3 parameter, which increased by 53% compared to initial level (See a Table). In this period AP level in experimental animals was statistically significantly higher (by 23%) compared to initial value — 133.6 ± 3.34 versus 108.32 ± 2.54 mm HG.

Heart rate variability values in rats in remote post-reperfusion period (M±m)

Parameters	Initial values	After 60 days of PRP
R-Rm, ms	157.24±7.21	151.4±9.68
CV, %	3.24±0.34	3.54±0.75
SDNN, ms	5.16±1.25	5.29±1.08
HF, NU	4.32±0.46	4.46±0.55
LF, NU	3.86±1.16	3.9±1.11
VLF, NU	5.47±1.32	5.76±1.07
TP, NU	6.15±1.13	6.17±1.07
N2	11.40±2.74	11.2±2.75
N3	19.40±4.67	29.7±4.27*
N4-6	46.20±5.72	41.5±3.43

* — differences are statistically significant compared to initial values, $p < 0.05$.

Discussion. The technique of division of ANP granules into certain types and their calculation under electron microscope is informative and sensitive that appears to be essential due to the lack of accurate and specific methods to study cardiac natriuretic peptides now [11]. For the first time we studied the intensity of ANP accumulation and release in a remote PRP on a whole organism using a complex of methods: quantitative analysis of immunolabeled granules of secretory cardiomyocytes, morphological and physiological techniques to assess the condition of animals. The researchers do not have the agreement of opinion whether an increased number of secretory granules in atrial cardiomyocytes indicate high endocrine activity or, by contrast, prolonged release of granules [4, 11].

In our view, an increased number of A- and B-type granules by day 60 of PRP indicated an intense synthesis, accumulation and release of ANP during period, since there was concurrently revealed high AP level being a stimulating factor for peptide formation and excretion. It has been reported in literature that under hypertension in blood plasma there is increased ANP level [1, 12]. Researcher have shown on cell cultures, on isolated myocardial tissue samples the relationship between an increased ANP level and activity of fibroblasts, a peptide having an inhibiting effect on synthetic and proliferative function of cells [13]. Thus, active fibroblasts, and the increase of collagen fibers and ground substance in interstitial space revealed in the study indirectly supported our supposition on active synthesis and ANP release in a remote PRP. In the authors' opinion [14], who studied myocardial changes after ischemia/reperfusion, the increase of the content of connective tissue components occurs due to cardiomyocyte death and due to an increased synthesis of extracellular matrix in early PRP. Such structural and functional alterations in the course of time cause myocardial remodeling [15]. There was similar morphological picture in atherosclerosis in patients with chronic heart failure and type 2 diabetes mellitus [16, 17]. In this case ANP could have a cardioprotective effect determined by researchers in clinical practice and in experiments [18–20]. Extended elements of sarcoplasmic reticulum could appear as prerequisites for granule formation activation. Similar studies we performed

[7] in early PRP revealed an increase of ANP formation and release with concurrent dilatation of sarcoplasmic reticulum in cardiomyocytes that was explained by ANP synthesis stimulation through receptors associated with G-proteins (Go and Gq) activating Ca^{2+} -dependent K^+ -channels SK4 located on SPR in paranuclear area [1]. Applied HRV analysis and AP measurement enabled to conclude the following: within the first minutes of PRP a short-term AP increase and the activation of sympatho-adrenal, pituitary-adrenal and rennin-angiotensive systems had no effect on ANP synthesis and secretion in the right atrial myocytes. On 60th minute of PRP when the heart was functioning on intracardial level, high intensity of ANP synthesis, accumulation and secretion in atrial myocytes was associated with a stimulating effect of hypoxic and ischemic factors during this period [7].

After 60 days of PRP the initial rhythm is recovered according to HRV values, except N3 parameter reflecting tonic influence of vagus. The authors revealed a positive effect of ANP on rhythmogenesis. The effect is achieved by two mechanisms; the first one — indirect, through autonomic nervous system, by inhibiting sympathetic and stimulating parasympathetic activity; the second — through direct regulation of specific cardiac ion channels reducing inward calcium current. ANP actively released by the right atrial cardiomyocytes is certain to be a component of a complex reaction chain promoting cardiac resuscitation in a remote PRP.

Thus, the study of ANP in an early and a remote PRP at a whole organism level using a complex of elaborated techniques (immunocytochemistry of cardiomyocyte granules, HRV and other methods) enabled to contribute to the understanding of the functioning of a group of natriuretic peptides under cardiovascular pathology, that is sure to have scientific and practical value.

Conclusion. Quantitative analysis of immunolabeled granules with a complex of morphological and physiological methods indicates the intensification of atrial natriuretic peptide accumulation and release in rat atrial cardiomyocytes by the 60th day of post-reperfusion period.

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