Atrial and Brain Natriuretic Peptides of Secretory Cardiomyocytes in Salt Loading in Experiment

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The aim of the investigation is to assess the influence of salt load on atrial (ANP) and brain (BNP) natriuretic peptide production in granules of secretory cardiomyocytes in rats.

Materials and Methods. The experiments were carried out on 14 white out-bred male Wistar rats weighing 280–300 g. During the experiment all the animals were treated with standard-feed diet and had unlimited access to food and water. NaCl solution was introduced *per os* in the dose of 1 g per 1 kg of body mass during 14 days. Arterial pressure (AP) was measured noninvasively using a tail-cuff method. ANP and BNP production of atrial cardiomyocytes was studied by means of immunohistochemistry, transmission electron microscopy, immunocytochemistry. There was performed a morphometric analysis of granules containing peptides (A-type — "mature, storing" and B-type — "dissolving").

Results. Increase in the number of granules with ANP and decrease in those with BNP accompanied by elevated AP was revealed 14 days after NaCl intake as compared to intact animals.

Conclusion. Natriuretic peptides metabolism is regulated by various mechanisms. Early BNP release does not promote AP reduction due to compensatory mechanism disturbance in salt-induced arterial hypertension. Increase in ANP production occurs under the influence of renin-angiotensin-aldosterone system and elevated AP. The present data can indicate adaptive reaction in response to salt loading.

Key words: atrial natriuretic peptide; ANP; brain natriuretic peptide; BNP; salt loading.

Atrial (ANP) and brain (BNP) natriuretic peptides are bioactive substances secreted in the heart, which cause natriuretic, vasodilatory, hypotensive effects and are antagonists of renin-angiotensin-aldosterone system (RAAS) [1, 2]. The hormones have similar molecular structure, receptor apparatus and mechanism of action [3, 4]. Researchers have found that natriuretic peptide secretion into the blood increases in arterial hypertension (AH) and their plasma concentration directly correlates with the severity of cardiac dysfunction [5–8]. However, the role of NP in AH pathogenesis as well as the causes of development and the progress of this pathology are not completely clear [9, 10].

The interrelation of arterial pressure (AP) and salt load has been well studied. It has been noted that in some people and experimental animals salt intake can easily provoke AP elevation but it has no effect in others [11–13]. High sensitivity to NaCl intake is expressed as significant AP elevation and is a risk factor in the development of AH, myocardial infarction, hemorrhagic stroke [14, 15]. Revealing this sensitivity in a clinic enables patients to be administered most effective therapy within a short time. Experimental studies in this field are determined by the search for new criteria of early diagnosis and are of great current interest [16, 17].

The study of natriuretic peptide in salt loading contributes to understanding AH pathogenesis [18]. Most often ANP and BNP are studied using biochemical methods [8, 19–22]. However, their plasma concentration does not always coincide with the quantitative characteristics of peptides in the heart [23]. Using the method of counting immunolabeled granules of different types provides the possibility to evaluate the contribution of ANP and BNP to cardio-vascular balance control in pathologic conditions. It should be noted that the studies where both hormones are analyzed are the most relevant [24, 25].

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The aim of the investigation is to assess the influence of salt load on atrial and brain natriuretic peptide production in granules of secretory cardiomyocytes in rats.

Materials and Methods. The experiments were carried out according to the rules of Good Laboratory Practice on 14 white out-bred male Wistar rats weighing 280–300 g. The work was performed in full accordance with the Ethical Principles of the European Convention for Protection of Vertebrate Animals used with Experimental and other Scientific Purposes (the Convention took place in Strasbourg on 18.03.1986 and was confirmed in Strasbourg on 15.06.2006). During the experiment all the rats were treated with standard-feed diet, had unlimited access to food and water and were housed under conditions of twelve-hour dark/light period. The control group (n=8) included intact animals, in the experimental group NaCl solution was introduced to the rats *per os* in the dose of 1 g per 1 kg of body mass during 14 days. AP was measured noninvasively using NonInvasive Blood Pressure Meter LE5001 (Panlab, Spain) with a tail-cuff before salt administration and 14 days after it. The animals were removed from the experiment by decapitation method. The samples for further analysis were taken 14 days after AP measuring.

To confirm antibody response specificity there was performed immunohistochemical analysis of the right atrial tissue samples of intact rats. The samples were fixed in 10% neutral formalin for 48 h, washed in running water, dehydrated in ethyl alcohol of increasing concentrations and embedded in paraffin. Histological sections, 5–7 µm thick, were obtained on a sliding microtome Leica SM 2000R (Leica Microsystems, Germany). Antigen reactivation was carried out by method of thermal retrieval using a citrate buffer with pH=6.0 (Novocastra Laboratories, UK) [26]. For ANP visualization we used primary polyclonal antibodies to ANP, Rabbit anti-Atrial Natriuretic Factor (1-28) (rat) with 1:50 working dilution. Rabbit anti-Brain Natriuretic Peptide-32 (Rat) Serum with 1:100 working dilution (Peninsula Lab. Inc., Bachem, USA) was used for visualization of BNP. Detection was performed using Peroxidase Detection System (Novocastra Laboratories, UK) in accordance with the manufacturer's instructions. Negative control was carried out by exclusion of primary antibodies.

Electron microscopy of the right atrial tissue samples of intact and experimental animals was performed using standard methods. The samples were fixed in 2.5% phosphate-buffered solution of glutaric dialdehyde (pH=7.4) and in 1% solution of OsO_4 with subsequent embedding into the mix of Epon and Araldit [27]. ANP and BNP cell localization was revealed on ultrathin sections using the above-mentioned polyclonal antibodies to ANP and BNP. Protein A conjugated with colloidal gold (Protein-A/Gold, 15 nm; EM Grade, Electron Microscopy Sciences, USA) was used as second antibodies [9]. Reactions were performed separately for each peptide. Sections processed with only second antibodies served as control. They were uranyl acetate and lead citrate contrasted and analyzed under the electron microscope Morgagni 268D (FEI, USA) using AnalySIS software.

A quantitative analysis of the two types of granules with peptides in atrial cardiomyocytes (A-type — "mature, storing" and B-type — "dissolving") was performed by counting in fields of vision (38×38 µm) [24, 28].

The findings were statistically processed with Statistica 10.0 software using Wilcoxon test and Mann–Whitney U-test.

Results. After 14 days we observed changes in AP in experimental group as compared to the initial indexes: in some animals AP (n=3) elevated from 98 to 105 mm Hg, in others (n=3) it decreased from 98 to 84 mm Hg (p<0.05). The present study has involved the animals with significantly elevated arterial pressure (by 10% compared to intact ones) for further investigation.
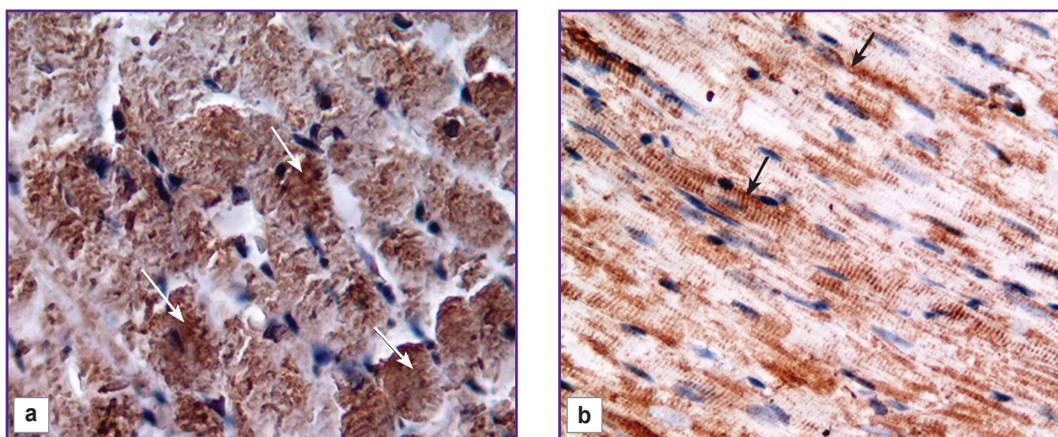

Figure 1. Immunohistochemical staining of atrial natriuretic peptide (a) and brain natriuretic peptide (b) in the right atrial cardiomyocytes of intact rats (arrows); ×400
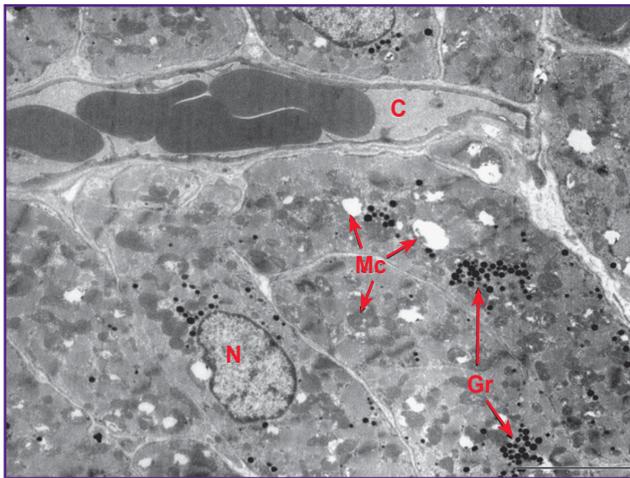
Figure 2. Ultrastructure of the right atrial myocardium after 14 days of salt loading: granules (Gr); capillary (C); mitichondria (Mc); nucleous (N); ×3,500

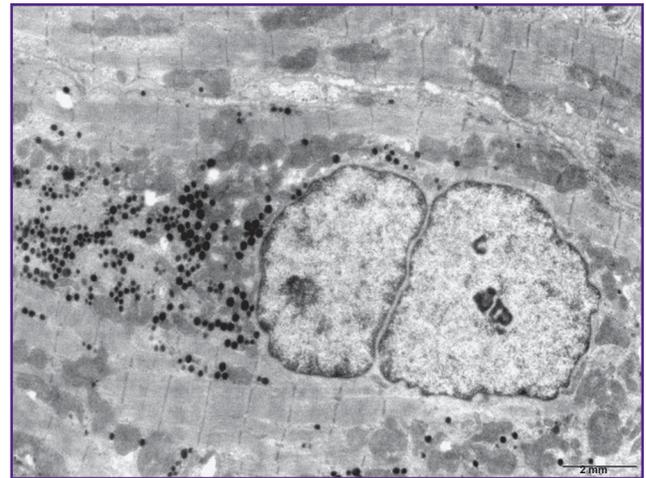**Figure 3.** Secretory granules in the right atrial cardiomyocyte after 14 days of salt loading; ×5,600

Proportion of A- and B-type granules containing atrial natriuretic peptide and brain natriuretic peptide in the atrial myocytes of rats after salt loading

Experiment groups	Granules containing ANP		Granules containing BNP	
	Type A	Type B	Type A	Type B
Intact animals (n=8)	65.75±19.50 63%	38.90±19.63 37%	16.0±5.43 68%	7.53±2.69 32%
14 days of salt loading (n=3)	117.96±33.5* 58%	88.83±28.06* 42%	6.42±3.42* 64%	3.65±2.79* 36%

* value differences are statistically significant compared to intact animals, p<0.05.

ANP- and BNP-immunoreactivity was found in the right atrium of intact rats with prevailing localization in the perinuclear space of cardiomyocytes. Notably, ANP-reaction (Figure 1 (a)) was more intense than that of BNP (Figure 1 (b)).

Electron-microscopic analysis of the right atrium of experimental animals showed the following changes in cardiomyocyte structure. Euchromatin with ill-defined aggregation to the periphery prevailed in the nucleus, mostly with no nucleoli. Karyolemma was smooth or with slight invaginations (Figure 2). A significant number of cytoplasmic granules was visually detected in sarcoplasm. Cisterns of sarcoplasmic reticulum were not extended. There prevailed swollen mitochondria with matrix clarification, cristae disorientation and fragmentation. Single giant mitochondria were also observed. Myofibrils were clearly defined in most cardiomyocytes. In general, sarcolemma had no visible changes, there were occasional folds and a few thinning areas. Moderate intercellular edema was detected (Figure 3).

After two weeks of salt loading there were detected morphometric changes in granules of the right atrial secretory cardiomyocytes, containing immunoreactive material. Granules with ANP of A-type were found to increase by 79% and B-type by 128%, their total number increased by 200%. Notably, the proportion of type A and B amounted to 58 and 42%, respectively, in intact animals it was 63 and 37%. The number of granules containing BNP decreased compared to the intact group: A-type by 60%, B-type by 52%, the total number decreased by 43%. The proportion of type A and B amounted to 64 and 36%, respectively, in intact animals it was 68 and 32% (See the Table).

Discussion. The revealed division of experimental animals into two groups according to AP changes confirms the data on salt-sensitivity reported in literature [29, 30]. Sensitivity to NaCl is caused by feature of kidney function, disturbance of sympathetic nervous system, prostaglandin synthesis, RAAS activity [31–33]. A group of salt-sensitive animals with elevated AP was studied in this investigation.

ANP and BNP response to the influence of various factors (ischemia, AH) and changes in their plasma concentrations are known to be similar in many ways [1, 9, 34]. This can be explained by the fact that both

peptides have similar structures and mechanisms of action, use the same cellular receptors for producing their effects, they are stored together in granules of secretory cardiocytes [24, 35]. However, scientific literature provides information about the differences in ANP and BNP properties in cardiovascular pathology [5, 6, 36]. In this study we have established diverse response of peptides in the right atrial myocytes of rats to salt.

In the experimental group increase in mature and dissolving forms of granules with ANP, compared to the intact animals, suggested active accumulation and release of the hormone. Therewith, A- and B-type proportion indicated processes shifting to its release. It has been established [37] that salt loading as well as AP elevation are factors promoting hypergranulation of atrial myocytes and ANP secretion. The other authors [38] have revealed 40% increase in AP, 351% increase in peptide granules and 100% increase in plasma level of the hormone in C57BL/6 mice (mouse model with human metabolic syndrome) after 9 weeks of NaCl intake. Clinical investigations demonstrate that salt loading activates RAAS in kidneys [39–42]. RAAS activation along with elevated AP is likely to be the main stimulus affecting ANP accumulation and release in experimental animals.

Analysis of immunolabeled granules with BNP in our investigation revealed decrease in the processes of its accumulation and release. According to the literature data concerning BNP activation in response to cardiovascular pathology development [43–45], this may speak of early secretion of the hormone. Apparently, it is intended to reduce AP. However, we observed its increase in the experimental group. Scientific literature and clinical practice still provide no answer to "the hormonal paradox" occurring in hypertension: high NP concentrations do not produce hypotensive effect [18]. Decrease in natriuretic peptide receptor density is considered to be one of the reasons [46] but this has not been proved sufficiently. Researchers have reported that normal compensatory mechanism of AP control by natriuretic peptides is suppressed by high-salt diet [38] due to changes in kidneys [47, 48]. Precisely this is the fact explaining the absence of hypotensive effect of early BNP release and high ANP level after 14 days of our experiment.

The authors of the study [49] have demonstrated an important role of Ca^{2+} ions in BNP response formation. Increased flow of Ca^{2+} ions to their matrix leading to cell death occurs through the mitochondrial calcium uniporter located in the mitochondrial membrane. BNP exerts an inhibitory effect on this channel, thereby preventing cardiomyocyte death [50]. The absence of marked changes in ultrastructure of the right atrium noted in the present study can be associated with a cardioprotective effect of BNP established in the earlier stages of the experiment. Simultaneously, destructive changes in

mitochondria may indicate disturbance of high-energy compound synthesis, which leads to decrease in BNP formation and release. In has been reliably established [3] that enhanced transport of Ca^{2+} ions promotes synthesis increase and formation of secretory granules with ANP and this is confirmed by the increase in total quantity of granules with ANP found after 14 days of our experiment.

Our study revealed various reactions of natriuretic peptides in salt loading. In salt-sensitive DOCA rats increase in the plasma level of ANP was detected after 2 weeks, while BNP level remained unchanged, with mRNA expression of both peptides in the myocardium being similar to that of the control [36]. According to the hypothesis, not only increased liquid volume, AP, hypertrophy and arrhythmia trigger synthesis and release of natriuretic peptides [51] but also oxygen gradient and myocardial energy metabolism [52, 53]. Apparently, salt load provokes shift of the above-mentioned factors in the right atrial cardiomyocytes, which has affected ANP and BNP variously in our experiments.

Thus, the obtained findings speak of diverse peptide response in granules of the right atrial myocytes of rats under the influence of salt. The observed decrease in BNP production and increase in ANP accumulation and release are likely to indicate adaptive reaction of the heart to salt diet.

Conclusion. Natriuretic peptide metabolism regulation is carried out by various mechanisms. The present study allows us to conclude that early release of brain natriuretic peptide does not promote AP reduction due to disturbance of compensatory mechanism in salt-induced arterial hypertension. Increase in atrial natriuretic peptide production occurs under the influence of renin-angiotensin-aldosterone system and elevated AP.

Conflicts of Interest. The authors have no conflicts of interest to declare.

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