

Influence of doxazosin on biosynthesis of S100A6 and atrial natriuretic factor peptides in the heart of spontaneously hypertensive rats

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Abstract

Hypertension frequently results in severe complications in cardiovascular system and histopathological changes in the heart. To better understand the cellular processes and signaling pathways responsible for the proper functioning of the heart, we decided to check whether doxazosin affects the density of structures containing S100A6 and atrial natriuretic factor in the heart of spontaneously hypertensive rats. The aim of this study is to find differences in the density of the structures containing S100A6 and atrial natriuretic factor in the heart of spontaneously hypertensive rats treated with doxazosin compared to untreated animals. Fragments of heart were collected from five spontaneously hypertensive rats and five spontaneously hypertensive rats receiving doxazosin for six weeks (dose 0.1 mg per 1 kg of body weight). On the paraffin sections S100A6 and atrial natriuretic factor peptides were localized in the heart using immunohistochemistry. Positive immunohistochemical reaction for S100A6 was observed in atrial and ventricular cardiomyocytes and in the coronary vasculature. In the heart of hypertensive rats treated with doxazosin the S100A6 immunoreactivity was significantly lower compared to untreated animals. Immunodetection of atrial natriuretic factor in the heart of rats confirmed presence of peptide in atrial myocardium. Delicate atrial natriuretic factor-immunoreactivity was observed also in few ventricular cardiomyocytes. The atrial natriuretic factor-immunosignal was significantly weaker in hearts of hypertensive rats receiving doxazosin compared to spontaneously hypertensive rats untreated. Since we found that doxazosin reduces the levels of S100A6 and atrial natriuretic factor peptides in the heart of spontaneously hypertensive rats, it can be assumed that cardiovascular disorders that occur in hypertension may be associated with disturbances of cellular processes and signaling pathways.

Keywords: S100A6, ANF, heart, rat, spontaneously hypertension, doxazosin

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Introduction

Hypertension is one of the most prevalent diseases worldwide. It was estimated that approximately 36–56% of the middle-aged population suffers from elevated blood pressure values.¹ The disease frequently results in severe complications in cardiovascular system, such as heart failure, cardiac ischemia or myocardial infarction.² Hypertension leads to histopathological changes in cardiac tissue including hypertrophy, fibrosis and inflammation, as well as remodelling of heart and vascular wall.³ In addition, abnormalities in myocardial mechanics were observed in patients with hypertension.⁴ A comparative evaluation of cardiomyocytes contractility in hypertensive and normotensive rats demonstrated that under hypertension conditions

cardiac muscle cells contracted much slower and with lower force.⁵ This pathological state contributes also to more frequent cardiomyocytes apoptosis.⁶

Function of cardiac muscle cells is strictly dependent on calcium ions, which play a major role in the activation of signal transduction pathways and regulate contractile performance. There are various calcium-binding proteins determining intracellular calcium cycling. Among them, increasing importance is attributed to S100 protein family. S100 family comprises low molecular proteins with two EF-hand type calcium-binding domains. Several members such as S100A1, S100A2, S100A6, and S100B were identified in heart tissue.^{7–9}

Ultrastructural localization of S100A1 in cardiomyocytes demonstrated that this peptide interacts with sarcoplasmic ryanodine receptor and sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA 2) – receptors responsible for Ca^{2+} transients during contraction cycle.^{8,9} Studies on mice and rabbits indicated that overproduction of S100A1 enhances cardiomyocytes contractility, whereas inactivation of S100A1 gene results in diminished contraction of cardiac muscle cells.^{10–12} Considering that mice with a deletion of S100A1 gene had elevated systolic blood pressure, it might be assumed that this protein is involved in hypertension-associated impaired functionality in cardiomyocytes.^{13,14}

Another member of the S100 family, S100A6 play a significant role in the regulation of cardiomyocytes differentiation and proliferation.¹⁵ It was indicated that S100A6 has a beneficent effect on cardiac muscle cell viability. Most et al.¹⁶ show that S100A1 prevents cardiac muscle cells death by activation of extracellular signal-regulated protein kinase 1/2 (ERK1/2). Tsoporis et al.^{17,18} demonstrated that S100A6 reduced cardiac myocytes loss induced by tumor necrosis factor-alpha (TNF- α) treatment. In consecutive studies, the authors found that S1006 attenuates cardiomyocytes hypertrophy and apoptosis in mice with myocardial infarction.

Beside impairment of myocardium contractility, hypertension also disturbs heart endocrine function. Larochelle et al.¹⁹ indicated that patients with essential and renovascular hypertension have elevated atrial natriuretic factor (ANF) concentration in blood. Increased ANF plasma content was also noticed in rats subjected to different hypertension models.^{20,21} Furthermore, studies of hypertensive animals revealed enhanced ANF content in heart homogenates.^{20,21} Considering that fluctuation of calcium ions in myocytes changes expression of ANF, it might be suspected that S100 proteins will affect ANF biosynthesis. This assumption was confirmed by Most et al.¹⁶ and Pleger et al.¹⁴ who demonstrated that S100A1 gene transduction weakened the rise of ANF production occurring after myocardial infarction.

Because of high mortality of hypertensive patients, researchers and clinicians keep on making every effort to improve the effectiveness of treatment of elevated blood pressure. Doxazosin – an antagonists of α 1 adrenoreceptors – is one of the recently launched medication to treat hypertension. It was found that doxazosin decreases the activity of sympathetic nervous system, causes relaxation of vascular smooth muscle, and thus lowers blood pressure in patients with essential hypertension.²² Unfortunately, doxazosin increased the risk of chronic heart failure in patients.²³ Moreover, in vitro studies demonstrated that doxazosin induces cardiomyocytes apoptosis.²⁴ Given that cardiomyocytes isolated from mice with deleted gene for α 1 adrenoreceptors had faster contraction rates, it might be suspected that block of those receptor by doxazosin could affect cardiac contractility.²⁵ This issue has not been, however, explored so far.

Up to now, there are no reports concerning localization of S100A6 and ANF in heart of hypertensive rats nor influence of doxazosin treatment on proteins distribution in cardiac tissue. For this reason, it appears worth undertaking

the immunohistochemical identification and evaluation of, S100A6- and ANF-positive structures in heart of rats with spontaneous hypertension with and without doxazosin treatment.

Materials and methods

Experimental model

Male spontaneously hypertension rats were purchased from Polish Mother's Memorial Hospital Research Institute in Lodz, Poland. All experimental procedures involving animals and their care were approved by local authorities and conducted in conformity with the national and international laws and Guidelines for the Use of Animals in Biochemical Research.²⁶ Study assumptions, aim, schedule and model of animal treatment were approved by the Senate Committee for Supervision of Experiments on Humans and Animals, Medical University of Bialystok Nr 2001/16.

The study was performed on 10 young male Wistar rats (6 weeks of age), whose body weight at the beginning of the experiment was within 180–200 g (the mean body weight: 190 ± 10 g). The animals were kept in lighted and ventilated conditions with room temperature and maintained day and night rhythm. The rats had a free access to standard granulated chow, and drinking water was available but were fasted overnight (16–18 h) before the experiment.

The animals were divided into two equal groups: control groups – five spontaneously hypertensive rats (SHR) (180 ± 6) and experimental group – five SHR treated with doxazosin (0.1 mg/1 kg b. w. in drinking water per day), similar in terms of baseline parameters. After six weeks, the experiment rats were anesthetized by pentobarbital, administered interperitoneally at a dose of 50 mg/kg; then the hearts were immediately collected. Subsequently, the hearts were fixed in Bouin's fluid for 24 h at $+4^\circ\text{C}$, and processed routinely for embedding in paraffin. Sections were cut at $4 \mu\text{m}$ in thickness, and processed by immunohistochemistry for S100A6 and ANF detection.

Identification of S100 proteins and ANF by immunohistochemical methods

Immunohistochemistry was performed using the EnVision Plus-HRP Detection Kit (K4011 Dako Denmark A/S, Produktionsvej 42, DK-2600 Glostrup) for rabbit antibody. Paraffin-embedded sections were deparaffinized and hydrated in pure alcohols. For antigen retrieval, the sections were subjected to pretreatment in a pressure chamber for 1 min at 21 psi at 125°C , using the Target Retrieval Solution with pH 6.0 (S 2369 Dako Denmark A/S, Produktionsvej 42, DK-2600 Glostrup) for S100A6 and using Target Retrieval Solution with pH 9.0 (S 2367, Dako Denmark A/S, Produktionsvej 42, DK-2600 Glostrup) for ANF (S 2367 Dako Denmark A/S, Produktionsvej 42, DK-2600 Glostrup). After being cooled to room temperature, sections were incubated with the Peroxidase Blocking Reagent (S 2001 Dako Denmark A/S, Produktionsvej 42, DK-2600 Glostrup) for 10 min, to block endogenous peroxidase activity. Subsequently, sections were incubated with primary

antibody for S100A6 (purchased from Nencki Institute of Experimental Biology, produced in-house) and for ANF (polyclonal rabbit ANF antiserum, H-005-24, purchased at the Phoenix Pharmaceuticals, Inc., Mountain View, CA). The antisera for S100A6 and ANF were previously diluted in Antibody Diluent (S 0809 Dako Denmark A/S, Produktionsvej 42, DK-2600 Glostrup) in a ratio of 1:500 and 1:2000, respectively. Sections with S100A6 antibody were incubated overnight at 4°C in a humidified chamber, whereas incubation with ANF-antibody lasted 1 h and was performed at room temperature. The procedure was followed by incubation with secondary antibody (conjugated to horseradish peroxidase-labelled polymer). The bound antibodies were visualized by 1-min incubation with liquid 3,3'-diaminobenzidine substrate chromogen. The sections were finally counterstained in hematoxylin QS (H-3404, Vector Laboratories; Burlingame, CA), mounted and evaluated under light microscope. Appropriate washing with Wash Buffer (S 3006 Dako Denmark A/S, Produktionsvej 42, DK-2600 Glostrup) was performed between each step. Negative control was carried out by incubating sections with the diluent and normal serum instead of the primary antiserum. All the performed control reactions gave negative results, and positive control was conducted for specific tissue recommended by producers.

Microscopic and quantitative analysis

Five rats were used for each studied group and five specimens of each antibody of each animal were observed and photomicrographed under the Olympus BX41 light microscope, with a digital camera (Olympus DP12) and a standard morphometric program (NIS-Elements Advanced Research software of Nikon) installed on computer.

The results of immunoreactive structure with S10A6 and ANF expression were searched for and observed their location.

From all heart sections, five randomly selected microscopic fields (each field of 0.785 mm², magnification of 200× (20× the lens and 10× the eyepiece)) were documented. In each analyzed image of heart, the width of 25 randomly selected cardiomyocytes was measured. In software for image analysis was also measured intensity of immunohistochemical reaction with S100A6 and ANF antibody. Intensity of immunohistochemical reaction was determined using 0 to 256 gray scale level, where a completely black pixel got a value of 0, whereas one with a value of 256 is completely white or bright.

Statistical analysis

All presented data were statistically analyzed by means of software computer package Statistica Version 10.0. The corresponding mean values were computed automatically; significant differences were determined by Student's *t*-test; *p* < 0.05 was taken as the level of significance.

Results

The antisera against S100A6 and ANF gave positive results in the hearts of all rats studied; however, the density of

S100A6- and ANF-containing structures and intensity of reactions varied between the groups studied.

S100A6 protein was identified in atrial and ventricular myocardium. The S100A6-immunoreactivity was more intense in atrial cardiomyocytes than in ventricular ones (Figure 1 (a) to (d)). In S100A6-immunopositive cardiomyocytes, immunosignal was the most intense in the vicinity of the cell nucleus. S100A6 protein was also found in endothelial cells and smooth muscle cells of coronary vasculature (Figure 1 (e) and (f)). In the hearts of hypertensive rats, the immunoreaction in cardiomyocytes was strong (atrium) or moderate (ventricle) (Figure 1(a) and (c)), while in rats which had undergone doxazosin treatment the intensity of S100A6-staining in cardiac muscle cells was weak (Figure 1(b) and (d)). Similarly, in rats receiving doxazosin a less intense S100A6-immunosignal in vessels supplying the heart wall was noticed (Figure 1(f)) when compared to untreated SHR (Figure 1(e)).

Immunohistochemical detection of ANF in the hearts of rats revealed its presence in atrial cardiomyocytes (Figure 2(a) and (b)). In the cytoplasm of atrial muscle cells darkly stained secretory granules were observed. A weak ANF-immunosignal was also noticed in a number of ventricular cardiomyocytes (Figure 2(c) and (d)). In the heart of SHR, numerous atrial muscle cells displayed strong immunostaining for ANF (Figure 2(a)). In the rats receiving doxazosin the presence of ANF was observed in a smaller number of atrial cardiomyocytes and the intensity of immunohistochemical reaction was significantly lower (Figure 2(b)) when compared to the animals not treated with doxazosin.

Computer image analysis confirmed weakening of the immunohistochemical reactions for S100A6 and ANF in the hearts of rats treated with doxazosin compared to drug-naive SHR (Table 1).

Morphometric studies showed a smaller diameter of cardiomyocytes of SHR treated with doxazosin compared to the myocardial cells of hypertensive rats receiving no medication (Table 1).

Discussion

Despite extensive research into the pathogenesis of hypertension and disease-related organ damage, the mechanisms leading to complications in the cardiovascular system of hypertensive patients are still not fully understood.

Myocardial contractile performance and the survival of cardiomyocytes are determined by calcium-binding proteins belonging to the S100 protein family. Recent studies indicate that cardiomyocytes function is also modulated by ANF which reduces the proliferation and contraction ability of these cells. However, it is clear from the review of the available literature that there are no reports evaluating changes in the distribution of S100 proteins and ANF in hypertensive heart disease. Clinical and experimental data show that doxazosin, which has recently been used in hypertension treatment, significantly affects cardiac action but to date no research on whether the drug influences the expression of the aforementioned peptides in cardiac tissue has been conducted.

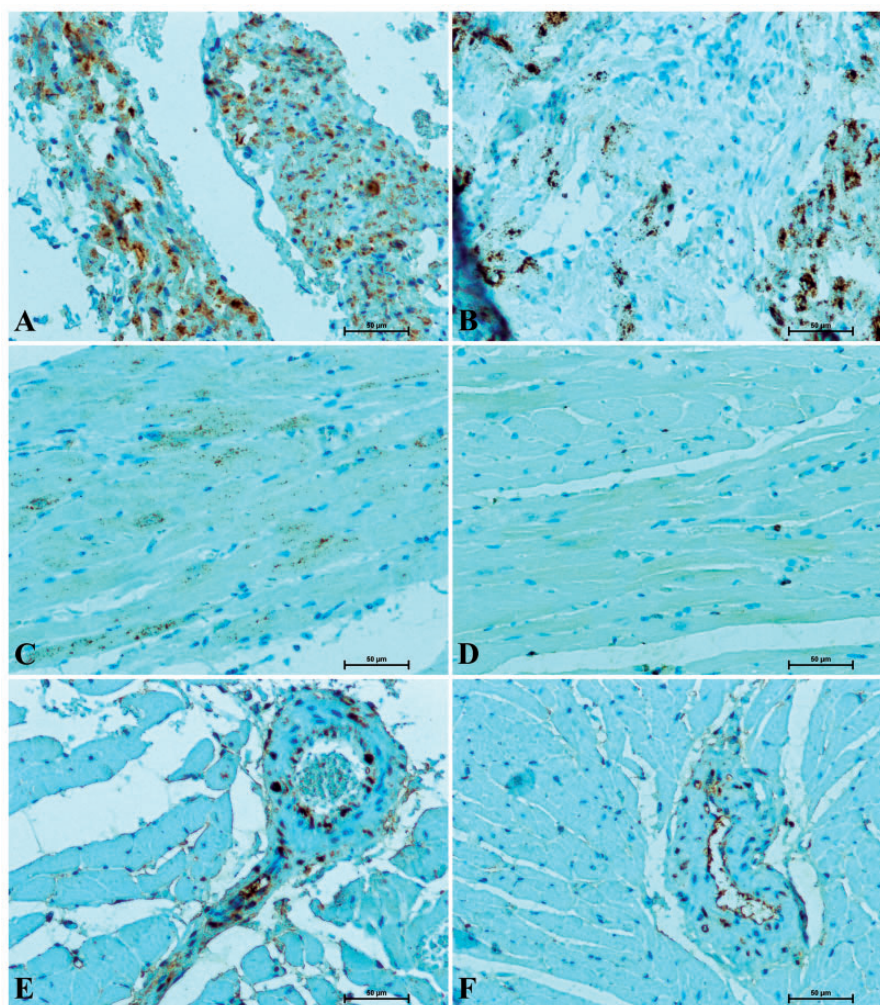


Figure 1 Immunodetection of S100A6 in heart of rat: (A) SHR – strong S100A6-reactivity in the atrial myocardium; (B) SHR with doxazosin treated – less numerous atrial muscle cells stained with anti-S100A6; (C) SHR – immunoreactivity with S100A6 in ventricular cardiomyocytes; (D) SHR receiving doxazosin – considerably weaker S100-immunoreaction in ventricular myocardium; (E) rat with spontaneous hypertension – intense reaction in the wall of coronary vasculature; (F) SHR with doxazosin treated – delicate signal for S100A6 in vessels supplying heart wall. (A color version of this figure is available in the online journal.)

These are the first studies concerning the immunohistochemical identification and comparative assessment of the S100A6 and ANF peptides in the hearts of rats with spontaneous hypertension treated and untreated with doxazosin.

Immunolabelling of S100A6 protein in rat hearts gave a positive reaction in atrial and ventricular cardiomyocytes and coronary vasculature in all the studied rats. The present studies demonstrate that the blockage of α_1 -adrenoceptors with doxazosin in SHR significantly lowered S100A6 immunoreactivity in heart tissue. Our findings, and studies by Tsoporis et al.^{17,27,28} who showed that the stimulation of the α_1 -adrenergic receptors caused an increase in the biosynthesis of S100 protein in rat cardiomyocytes, suggest a significant role of α_1 -adrenoceptors in the regulation of S100A6 gene expression.

The changing hemodynamic conditions in hypertension lead to the remodelling of the heart wall and cardiac hypertrophy. There is evidence that hypertension-associated heart hypertrophy might be invoked by the activation of the α_1 -adrenergic receptors. During hypertension, the

level of norepinephrine (NE), which stimulates α_1 -adrenoceptors, is significantly increased. Tsoporis et al.²⁸ demonstrated that α_1 -adrenoceptors agonist phenylephrine (PE) induced the biosynthesis of the skeletal α -actin (skACT) and β -myosin heavy chain (β -MHC) in rat cardiomyocytes, and resulted in an increase in the number of these cells. Other researchers have observed that the inhibition of α_1 -adrenoceptor with doxazosin attenuated cardiac hypertrophy in rats with ventricular pressure-overload.²⁹ A reduction of ventricular hypertrophy after doxazosin treatment was also observed in patients with essential hypertension.³⁰

Our results confirm a reduction in the diameter of cardiac muscle cells in rats with spontaneous hypertension treated with doxazosin compared to untreated SHR.

Literature data suggest that S100A6 protein might be involved in the adaptive processes limiting progression of heart hypertrophy in hypertension. Tsoporis et al.^{18,28} stated that S100A6 attenuated cardiomyocytes hypertrophy induced by PE. The weakening of S100A6-reaction in the hearts of SHR receiving doxazosin, demonstrated by the current study, might be related to the properties of the

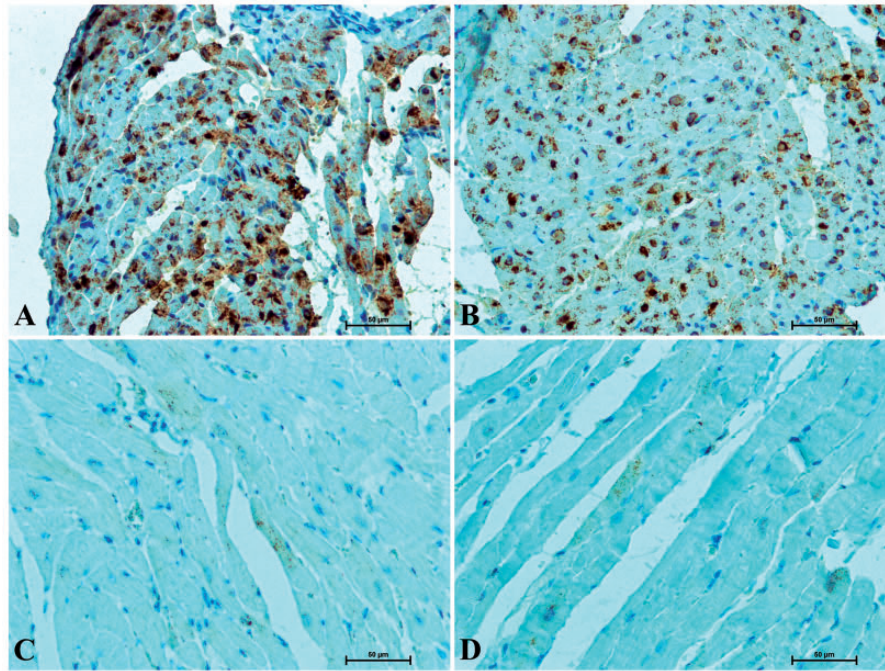


Figure 2 Positive ANF-immunostaining in heart of rat (A) SHR – very strong immunosignal in majority of atrial cardiomyocytes, (B) SHR with doxazosin treated – less numerous atrial muscle cells with ANF-immunoreactivity; (C) SHR – delicate ANF-reaction in some of ventricular cardiomyocytes; (D) SHR receiving doxazosin – single ventricular muscle cells with weak ANF-immunoreactivity. (A color version of this figure is available in the online journal.)

Table 1 Intensity of immunohistochemical reaction with S100A6 and ANF antibody and the width (μm) of cardiomyocytes in hearts of control rats, untreated doxazosin SHR and doxazosin treated SHR (mean \pm SD)

Group of rats	Width of cardiomyocytes (μm)	Intensity of IR for S100A6	Intensity of IR for ANF
SHR rats	13.5 \pm 1.76	91.7 \pm 20.00	59.4 \pm 28.30
SHR rats treated with doxazosin	11.9 \pm 2.30	144.9 \pm 24.48	64.7 \pm 31.09
p	<0.0001	<0.0001	0.0082

IR: immunoreactivity.

drug associated with the inhibition of the heart cells hypertrophy.

The *in vitro* studies indicate that doxazosin induces cell apoptosis by the upregulation of Bax, caspase-3, caspase-8 gene and the downregulation of the Bcl-2 gene.³¹ On the basis of the latest reports it might be assumed that S100A6 is implicated in this process. Joo et al.³² showed that S100A6 protein promoted cell death by increasing caspase-3 level. Slomnicki et al.³³ demonstrated that S100A6 increased the transcription of p53 protein and enhanced cell sensitivity to oxidative stress. On the other hand, other researchers have demonstrated the beneficial effect of S100A6 on cardiomyocytes viability. Tsoporis et al.¹⁷ noticed that S100A6 prevented apoptosis of cardiomyocytes caused by TNF α . Some authors showed that the induced overexpression of S100A6 protein decreased cardiomyocytes loss in rats with experimental myocardial infarction.¹⁸ The decreased intensity of S100A6-immunostaining in the hearts of SHR treated with doxazosin observed in the present study, might be explained by the compensatory mechanism attenuating cardiomyocytes death, or might be one of the mechanisms of the proapoptotic action of doxazosin. This matter requires

further research in order to gain a better understanding of the role of S100A6 protein in determining cardiomyocytes viability under physiological and pathological conditions.

Immunohistochemical identification of ANF in the hearts of rats confirmed the presence of the peptide in atrial myocardium. A weak ANF-reaction was also observed in a number of ventricular cardiomyocytes. The ANF-immunosignal was significantly weaker in the hearts of hypertensive rats receiving doxazosin in comparison to untreated SHR. Our findings are consistent with reports by Sakata et al.³⁴ who indicated that the blockade of α_1 -adrenoceptor with doxazosin decreased ANF production.

Multiple mechanisms including the increased activity of renin-angiotensin-aldosterone system (RAA) and the activation of the hypothalamic-pituitary-adrenal axis (HPA-axis) are involved in the pathogenesis of hypertension. Both systems participate not only in stress response, vascular resistance regulation and water-mineral balance, but also modulate ANF biosynthesis in the heart.

Experimental studies have demonstrated that the activity of the HPA axis is regulated by α_1 -adrenoceptors. Shimizu³⁵ stated that agonists (NE and PE) of

α_1 -adrenoceptors induced the secretion of adrenocorticotrophic hormone (ACTH), whereas antagonists (phentolamine and phenoxybenzamine) of α_1 -adrenoceptors had the opposite effect. Similar findings are presented in reports by Feldman and Weidenfeld,³⁶ Handley and Mithani,³⁷ Yorimitsu et al.³⁸ Jager et al.³⁹ stated that doxazosin had a negative effect on aldosterone secretion by adrenocortical cells porcine. Mulaturo et al.⁴⁰ found that an intravenous administration of doxazosin to patients with primary aldosteronism significantly lowered plasma aldosterone concentration. Considering the above, the observed reduction in ANF-immunoreactivity in the hearts of SHR treated with doxazosin may result from the negative action of the drug on HPA axis activity and the secretion of adrenocortical hormones.

The principal mechanisms stimulating ANF secretion are associated with an increase in sodium concentration in the blood and hypervolemia. It has been documented that α_1 -adrenoceptors influence the renal blood flow, urine volume, sodium and potassium excretion. Elhawary and Pang⁴¹ stated that injecting rats with α_1 -adrenergic stimulators caused antidiuresis and antinatriuresis, while specific α_1 -adrenoceptor antagonists abolished this effect. The enhanced tubular Na^+ and water reabsorption after the activation of α_1 -adrenoceptors has also been demonstrated in dogs⁴² and rabbits.⁴³ Therefore, it might be assumed that a decrease in the intensity of ANF-immunosignal in the hearts of SHR receiving doxazosin observed in our study may be associated with the action of doxazosin on renal function and fluid volume.

The results of numerous studies point to the important role of inflammation in the pathogenesis of hypertension. It has been also found that several interleukins enhance the production of ANF in atrial myocytes. Considering that α_1 -adrenoceptors are expressed in leucocytes, it might be assumed that these receptors are involved in the regulation of the immune response in physiological and pathological conditions. Grisanti et al.,⁴⁴ documented that the activation of α_1 -adrenoceptors stimulates immune cells and secretion of proinflammatory cytokines. Other studies have revealed that prazosin α_1 -adrenoceptor antagonist significantly reduces interleukin-6 level in patients subjected to physical exertion.⁴⁵ Given the above, a weakened ANF-immunoreaction in hypertensive rats receiving doxazosin might be explained by suppression of the immune system after drug treatment.

The α_1 -adrenoceptor regulates contractile performance of cardiac muscle cells. The α_1 -adrenergic stimulation significantly increases contraction of cardiomyocytes. The blockage of α_1 -adrenoceptor inhibits calcium outflow from the endoplasmic reticulum and results in impaired functioning of cardiac muscle cells. It has been indicated that ANF significantly reduces cardiomyocytes contractility. Delaflotte et al.⁴⁶ and Bilzer et al.⁴⁷ showed that ANF had the ability to antagonize vasoconstrictive effect of α_1 -adrenergic agonists in rats. In view of the above, reduction of ANF - immunoreactivity in the hearts of SHR treated with doxazosin - is possibly a result of compensatory mechanisms limiting further deterioration of cardiac function after receiving the medication.

The present report suggests that S100A6 and ANF might be involved in the protection of cardiac cells in hypertension.

Authors' contributions: IK, ZP, AF, MM participated in the study design; IK, ZP, AF and MM conducted the experiments, data collection and analysis of the data; IK wrote the manuscript; ZP contributed to manuscript preparation and performed statistical analysis of those studies. All authors participated in interpretation of the studies and review and approved the final manuscript.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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