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Furosemide modifies heart hypertrophy and glycosaminoglycan myocardium content in a rat model of neurogenic hypertension

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ABSTRACT

Hypertension is a major risk factor for atherogenesis and heart hypertrophy, both of which are associated with specific morphological and functional changes of the myocardium. Glycosaminoglycans (GAGs) are complex molecules involved both in tissue morphology and function. In the present study, we investigated the effects of neurogenic hypertension and subsequent antihypertensive treatment with furosemide, on heart hypertrophy and the content of GAGs in the myocardium. Neurogenic hypertension was achieved in male Wistar rats by bilateral aortic denervation (bAD). At days 2, 7 and 15 after surgery, animals were sacrificed and the hearts were dissected away, weighted, and homogenized. Total GAGs were assessed by measuring the uronic acid content colorimetrically and individual GAGs were isolated and characterized by enzymatic treatment, with GAG-degrading enzymes, using electrophoresis on polyacrylamide gradient gels and cellulose acetate membranes. In bAD-animals blood pressure, blood pressure lability, heart rate and heart weight were significantly increased 15 days postoperatively. These effects were prevented by treatment with furosemide. Major GAGs identified in the heart were chondroitin sulphates, heparin (H), heparan sulphate (HS) and hyaluronic acid. The content of uronic and the relative content of H and HS in the heart in bAD animals significantly decreased from day 2 to day 15 postoperatively. Furosemide prevented the bAD induced decrease in GAG content. Considering that H and HS are potent inhibitors of cardiomyocyte hypertrophy, our results indicate that heart hypertrophy induced by neurogenic hypertension may be associated with decreases in the relative content of heparin and heparan sulphate in the heart.

1. Introduction

Hypertension is a major cause of heart hypertrophy associated with specific morphological changes of the myocardium, such as fibrosis, and is closely related to a complex cascade of events leading to atherogenesis (Ross, 1993). Together, these structural changes attenuate the functional capacity of the cardiovascular system.

Among different molecules involved in the process of atherogenesis, the glycosaminoglycans (GAGs) constitute key elements in the formation of atherosclerotic lesions (Wight, 1985). GAGs constitute a group of complex macromolecules that exist both on the cell surface and free within the extracellular matrix, playing a pivotal role in cell function and connective tissue formation. GAGs of extracellular matrix provide structural links between fibrous and cellular elements, contribute to viscoelastic properties, regulate permeability and retention of plasma components within the matrix (Wight, 1985;Wight, 1989), inhibit vascular cell growth (Fritze et al., 1985), affect haemostasis and platelet aggregation (Vijayagopal et al., 1980) and interact with lipoproteins (Iverius, 1972).

Treatment of hypertension targets not only to the reduction of blood pressure, but also to the prevention of cardiovascular events. Although loop diuretics, such as furosemide (FUR), have long been applied antihypertensive agents with excellent results on the basis of cardiovascular morbidity and mortality, the effects of diuretics on cardiac hypertrophy are poorly understood (Kim et al., 1996).

Neurogenic hypertension animal models have been successfully employed to elucidate aspects of the pathophysiology of hypertension (Krieger, 1964; Singewald et al., 1997; Kouvelas et al., 2006, 2009a;). However, there is limited data regarding the effect of neurogenic hypertension on the pathogenesis of cardiac

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hypertrophy or vascular wall damage and the involvement, therein, of extracellular matrix molecules, such as GAGs. In the present study we hypothesized that heart hypertrophy and arterial stiffness promoted by neurogenic hypertension in a rat model of bilateral aortic denervation (bAD), may be due to alterations of GAGs, and that these responses could be limited by the application of diuretics.

2. Materials and methods

The study was approved by the Research Ethical Committee of the School of Medicine, Aristotle University of Thessaloniki and it conforms to the provisions of the Declaration of Helsinki (as revised in Brazil in 2013). Also the study has been conducted in a manner that does not inflict unnecessary pain or discomfort upon the animals, as outlined by the United States Public Health Service Policy on Humane Care and Use of Laboratory Animals (2002) and the Guide for the Care and Use of Laboratory Animals (1996), prepared by the National Academy of Sciences' Institute for Laboratory Animal Research.

2.1. Experimental setting

Male Wistar rats (aged 10–12 weeks old, weighting 230–300 gr) were single housed, and maintained in a climate-controlled room, on a 12-h light-dark cycle and allowed food and water ad libitum. Animals were randomly assigned into 4 groups, as follows: (a) control group (n=14); (b) sham operated group: animals subjected to bilateral cervical incision (n=30); (c) neurogenic hypertensive group: animals subjected to bilateral aortic denervation (bAD) (bAD; n=30); and (d) neurogenic hypertensive group treated with furosemide: animals subjected to bAD, followed by subcutaneous furosemide administration, 3 mg twice daily (bAD+Fur; n=30) (Table 1).

Rats were anesthetized with ketamine (50 mg/kg, intraperitoneally) and suitably fixed in a supine position. An extensive (3–4 cm) midline cervical incision was made and the sternocleidomastoid muscles bilaterally were pushed aside, exposing the neurovascular sheath. The common carotid arteries and the vagal trunk were carefully isolated. bAD consisted of resection of the superior cervical ganglion and superior laryngeal nerves (Fink et al., 1980; el-Mas et al., 1994; Singewald et al., 1997; Kouvelas et al., 2009b;). Sham operation was performed under the same conditions, but no denervation was carried out, and the nerves remained intact. Successful denervation was attested by the slight heart rate reduction in response to intravenous injection of 4 g/kg phenylephrine after bAD (Kouvelas et al., 2006, 2009a, 2009b). The skin was apposed using 3–0 silk sutures and rats were allowed to recover spontaneously after denervation. Animals in sham, bAD and bAD+furosemide groups were sacrificed 2, 7 and 15 days postoperatively (8 animals in each subgroup) and their hearts and aortas were dissected away. (Table 1) The hearts were extracted, weighted (wet weight), washed in saline, dissected and homogenized. Lipids were extracted using a mixture of chloroform / methanol and the dry defatted weight of the hearts was measured. Animals in control group were sacrificed and treated as above.

2.2. Data collection

2.2.1. Blood pressure monitoring

Blood pressure and heart rate monitoring was recorded by a tail cuff sphygmomanometer (IITC Life Science Systems) at predefined time frame (from 12:00 to 14:00) on the 2nd, 7th and 15th postoperative days. Furthermore, blood pressure lability was calculated as the coefficient of variation (standard deviation/mean) of blood pressure values (Alper et al., 1987; Kouvelas et al., 2009a). The standard deviation was calculated for each animal from 60 measurements carried out once per minute over a period of 1 h. Statistical analysis was carried out after logarithmic transformation.

2.2.2. Isolation and purification of total glycans

Heart tissues were cut into small pieces and homogenized by a Polytron homogenizer (five x10 sec bursts with 1 min intervals in ice), in a buffer solution containing 100 mM Tris–HCl, pH 8.0 and 1 mM $CaCl_2$. Homogenized samples were subjected to ultrasonication in a Clifton Ultrasonic bath (Nickel-Electro, Weston-Super-Mare, North Somerset, UK) (3 × 5 min), in order to disrupt the integrity of the tissue. Total GAGs were isolated and purified from the whole tissue homogenates as previously described (Papakonstantinou et al., 1995) and colorimetric determination of uronic acids was performed in each sample according to Bitter and Muir (Bitter and Muir, 1962).

2.2.3. Purification and identification of specific GAG populations from total GAGs was achieved by electrophoresis on cellulose acetate membranes (ECAM) and polyacrylamide gel electrophoresis (PAGE)

a. ECAM was performed using two μl of each total GAG solution, containing 4 μg of uronic acids, as previously described (Papakonstantinou et al., 2000).
b. PAGE. Linear gradient (4–20%) polyacrylamide gels were prepared and PAGE was performed using two μl of total GAG solution, containing 10 μg of uronic acids as previously described (Papakonstantinou and Misevic, 1993; Papakonstantinou et al., 2000). Commercially available standard GAGs, the molecular weight of which had been determined by analytical ultracentrifugation, were used as molecular weight markers (Papakonstantinou, et al., 2000)

2.2.4. Quantitation of the intensity of staining from ECAM and PAGE

The intensity of the staining was quantified by scanning densitometry using a computer-assisted image analysis program (1D

Table 1
Description of surgery, diuretic treatment, blood pressure measurements, GAGs determination and histological analysis per group (n=number of rats included in each group).

Groups	Surgery	Treatment	Blood Pressure measurements			Heart Dissection			
						GAG determination			Histological analysis
			Day 2	Day 7	Day 15	Day 2	Day 7	Day 15	Day 15
Control n=14	None	None	n=8	n=8	n=8	n=0	n=0	n=8	n=6
SHAM n=30	Sham operation	None	n=8	n=8	n=8	n=8	n=8	n=8	n=6
bAD n=30	ADN denervation	None	n=8	n=8	n=8	n=8	n=8	n=8	n=6
bAD+Furo n=30	ADN denervation	Furosemide	n=8	n=8	n=8	n=8	n=8	n=8	n=6