Spontaneous, L-Arginine-Induced and Spironolactone-Induced Regression of Protein Remodeling of the Left Ventricle in L-NAME-Induced Hypertension

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Running title: *Protein remodeling in L-NAME-induced hypertension*

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Summary

N^G-nitro-L-arginine-methyl ester (L-NAME)-induced hypertension is associated with protein remodeling of the left ventricle. The aim of the study was to show, whether aldosterone receptor blocker spironolactone and precursor of NO-production L-arginine were able to reverse the protein rebuilding of the left ventricle. Six groups of male Wistar rats were investigated: control 4 (4 weeks placebo), L-NAME (4 weeks L-NAME), spontaneous-regression (4 weeks L-NAME + 3 weeks placebo), spironolactone-regression (4 weeks L-NAME + 3 weeks spironolactone), L-arginine-regression (4 weeks L-NAME + 3 weeks arginine), control 7 (7 weeks placebo). L-NAME administration induced hypertension, hypertrophy of the left ventricle (LV), and the increase of metabolic, contractile and soluble and insoluble collagenous protein concentration. The systolic blood pressure and relative weight of the LV decreased in all three groups with regression, while the most prominent attenuation of the LVH was observed after spironolactone treatment. In the spontaneous-regression and L-arginine-regression groups the concentrations of individual proteins were not significantly different from the control value. However, in the spironolactone-regression group the concentration of metabolic, contractile and insoluble collagenous proteins remained significantly increased in comparison to the control group. The persistence of the increased protein concentration in the spironolactone group may be related to the more prominent reduction of myocardial water content by spironolactone.

Key words: collagen, L-NAME hypertension, L-arginine, spironolactone, regression of hypertrophy

Introduction

Left ventricular hypertrophy (LVH), although an adaptive mechanism compensating increased hemodynamic overload, is an independent risk factor of increased cardiovascular morbidity and mortality through higher incidence of heart failure, arrhythmias, myocardial infarction or stroke. It is believed that regression of LVH diminishes the incidence of cardiovascular risk (Devereux *et al.* 2004).

A number of drugs are being tested to determine their potential to reverse LVH (Mandarim-de-Lacerda and Pereira 2002, Klingbeil *et al.* 2003, Kojšová *et al*. 2006, Šimko and Paulis 2007). Nitric oxide was shown to have vasodilatative but also antiproliferative and antigrowth properties (Kolpakov *et al.* 1995, Šimko and Šimko 2000, Šimko 2007). The precursor of NO-formation L-arginine was shown to reduce blood pressure in human (Nakaki *et al.* 1990) and to reverse LVH in spontaneously hypertensive rats (Matsuoka *et al.* 1996), however it failed to prevent hypertension and LVH in L-NAME-induced hypertension (Šimko *et al.* 2005). Aldosterone is the growth stimulating factor, participating on the LVH and fibrosis development (Brilla *et al.* 1993). Spironolactone, the blocker of aldosterone receptor, reduced the extent of LVH and DNA concentration in L-NAME-induced hypertension in a preventive experiment (Šimko *et al.* 2007). Most importantly, spironolactone reduced mortality in patients with severe heart failure in the Randomised Aldactone Evaluation Study (RALES) when added to standard treatment (Pitt *et al.* 1999, Šimko *et al.* 2002), while antifibrotic potential of spironolactone was considered to play an important role in this benefit (Zannad *et al.* 2000).

In previous experiments, L-NAME was shown to induce hypertension, LVH and fibrosis of the LV (Pecháňová *et al.* 1997, Šimko *et al.* 2007). The aim of this

study was to show whether L-arginine and spironolactone are able to reverse the collagenous and non-collagenous protein remodeling of the LV in NO-deficient hypertension.

Methods

Animals and treatment

Male 12-week-old Wistar rats were randomly divided into six groups. In the control 4 (CT4, n=8) group the animals were sacrificed after 4-week placebo treatment and in the control 7 (CT7, n=8) group after 7-week placebo treatment. The L-NAME group (LN4, n=9), where 40 mg/kg/day L-NAME was given for 4 weeks, served for assessment of the development of L-NAME-induced alterations and was compared with CT4. In the residual three groups the regression of L-NAME-induced alterations was observed and was compared with the both LN4 and CT7 groups. In these groups the 4-week L-NAME-treatment (40 mg/kg/day) was replaced by 3 weeks of either placebo treatment in the spontaneous recovery group (SPR, n=9) or by administration of 1500 mg/kg/day L-arginine (Merck, Germany) in the L-arginine-induced recovery group (ARG, n=9), or by administration of 200 mg/kg/day spironolactone (Gedeon, Hungary) in the spironolactone-induced recovery group (SPI, n=9). Experimental design is given in Fig.1.

All animals were housed in individual cages at a temperature of 22-24°C and fed a regular pellet diet, with access to food and water *ad libitum*. L-NAME and L-arginine were given in tap water and spironolactone was mixed with methylcellulose as an emulsion and was applied via gavage twice daily. Moreover, all other animals were gavaged with placebo (methylcellulose) twice daily so that

handling conditions were preserved for all animals in the experiment. The investigation conformed on the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 8532, revised 1985).

Hemodynamic parameters

The systolic blood pressure (SBP) was measured weekly by non-invasive tail-cuff plethysmography (Hugo-Sachs Elektronik KG, Germany). After 6 weeks the rats were sacrificed by decapitation and the heart was excided. The body weight (BW), left ventricle weight (LVW) and right ventricle weight (RVW) were determined and RVW/BW as well as LVW/BW ratios were calculated.

Determination of left ventricle protein profile

The LV samples were rapidly weighed and transferred into precooled homogenization test tubes. They were frozen subsequently to -50°C. Tissue samples were later thawed, a 40-fold volume was achieved by adding 50 mmol/l sodium-potassium phosphate buffer, pH 7.4 containing 10 mmol/l EDTA and 1% Triton X, homogenized and centrifuged at 15 000 x g; supernatant was used for the determination of metabolic proteins (MP). The pellet was resuspended and fractions of contractile and collagenous proteins were obtained in a stepwise manner by extracting contractile proteins into a supernatant with phosphate buffer (100 mmol/l, pH 7.7, containing 1.1 mmol/l KCl). The pellet was shortly washed with 0.5 mol/l acetic acid then extracted with 0.5 mol/l CH_3COOH-pepsin concentration was kept in the range 1:100 - 1:50. After 24 h at 4°C, the extracts were centrifuged. The supernatant contained the fraction of soluble collagenous proteins. The pellet was further suspended in 1.1 mol/l NaOH and left for 45 min at 105°C. This fraction

contained insoluble collagenous proteins. The protein profile procedure yielded three basic fractions: 1) metabolic proteins (containing predominantly enzyme systems for aerobic and anaerobic substrate utilization) (Bass *et al.* 1988); 2) contractile proteins (complex of contractile, regulatory and modulatory proteins of myofibrils, 3) structural collagenous proteins (the fraction included collagens, elastins, proteoglycans and glycoproteins), which can be divided into two fractions: (a) soluble collagenous proteins constituted mainly by collagen I and III and (b) insoluble collagenous proteins including collagen aggregates, elastins and other proteins of the extracellular matrix. This methodological approach has been described in detail elsewhere (Pelouch *et al.* 1993, Pelouch *et al.* 1995, Pelouch *et al.* 1996). Protein concentration in individual fractions was determined according to Lowry *et al.* (1951) and expressed per g of tissue wet weight; protein content was expressed per total LV wet weight.

Statistical analysis

Values of individual parameters expressed in figures and tables are declared as mean ± SEM. Statistical evaluation was performed using the SigmaStat software program (revision STAT 32 2.0, Jandel GmbH, Erkrath, Germany).

Results

Blood pressure and weight of the heart

After four weeks of experiment, the SBP was 128 ± 3 mm Hg in the CT4 group. L-NAME administration elevated SBP by 32 % ($P<0.001$) *vs.* CT4 group. After seven weeks, the SBP was 124 ± 2 mmHg in the CT7. In all recovery groups

SBP decreased significantly (P<0.05) compared to the LN4 group, however, compared to CT7, SBP remained elevated (P<0.05) in the SPR and ARG group, respectively (Fig. 2).

After four weeks, the LVW/BW ratio was 1.32 ± 0.02 mg/g in CT4 group. In LN4 group the LVW/BW ratio increased by 21 % (P<0.05) *vs.* CT4 group. LVW/BW decreased significantly (P<0.05) in all groups with regression (SPR, Arg, SPI) *vs.* LN4 (Fig. 2, Tab. 1).

Protein composition of the left ventricle

In the LN4 group, the concentration of metabolic proteins (94.39 ± 3.74 mg/g) increased significantly (P<0.05) vs CT4 (82.69 ± 1.71 mg/g). The concentration of contractile proteins (97.88 ± 2.90 mg/g) was higher (P<0.05) in comparison with CT4 (88.92 ± 1.63 mg/g). In the SPR and ARG groups concentrations of both metabolic and contractile proteins were not increased compared to the CT7 group. However, after three weeks of spironolactone treatment, the concentration of both metabolic and contractile proteins remained increased (P<0.05) in comparison to the CT7 (Fig. 3).

Collagenous proteins in both soluble and insoluble fractions were increased in LN4 group (52.08 ± 2.02 mg/g and 18.26 ± 1.80 mg/g, respectively) *vs.* CT4 (44.77 ± 1.15 mg/g and 11.78 ± 1.09 mg/g, respectively). No significant changes of the concentration of soluble collagenous proteins were detected in any group with LVH regression. The regression of increased insoluble collagenous protein concentration was achieved in the SPR and ARG groups, while after spironolactone treatment the concentration of insoluble collagenous proteins remained significantly (P<0.05) elevated vs. CT7 (Fig. 4) (Table 2).

Discussion

Administration of L-NAME induced hypertension, hypertrophy of the left ventricle, and the increase of metabolic, contractile and soluble and insoluble collagenous protein concentrations. The relative weight of the left ventricle was reversed to the original level in all the three recovery groups. In the SPR and ARG groups concentration of metabolic and contractile proteins, as well as of collagenous proteins were not different from the control value. However, after three weeks of spironolactone treatment, the concentration of individual protein fractions remained increased in comparison to the control group.

L-NAME-induced hypertension is associated with the whole number of structural and functional alterations of the heart, kidney and vessels (Bernátová *et al.* 1999, Hropot *et al.* 1994, Kristek *et al.*2005, Okruhlicová *et al.* 2005, Pereira *et al.* 2004), however, the underlying mechanisms are not quite clear. NO-deficiency and excessive activation of the renin-angiotensin II-aldosterone system seems to play the most important role (Šimko and Šimko 2000). In our previous works, we tested several substances with respect to their potential protective effect on NO-deficient hypertension. Captopril prevented both hypertension and LV remodeling (Pecháňová *et al.* 1997), and spironolactone in a preventive experiment reduced hypertension, mass of the LV and DNA concentration (Šimko *et al.* 2007). On the other hand, simvastatin, despite reduction of blood pressure, did not prevent neither hypertrophy nor fibrosis of the LV and hypertrophy of the aorta (Šimko *et al.* 2004) and L-arginine had no effect on blood pressure and LV remodeling in L-NAME hypertension (Šimko *et al.* 2005).

There are only seldom data describing the regression of already developed changes induced by chronic L-NAME treatment. In the present study, the growth of myocardium was associated with the enhancement of pepsin soluble (i.e. newly synthesized collagen) and pepsin insoluble (i.e. collagen aggregates) collagenous protein concentration. The simultaneous increase in metabolic and contractile non-collagenous proteins after long-term L-NAME treatment may represent a potential adaptive response of the myocardium to increased hemodynamic demands. The cessation of L-NAME administration, followed by placebo, L-arginine or spironolactone treatment, resulted in the regression of hypertension and LVH in a three week period, while the LV mass was most prominently reduced with spironolactone. The concentration of contractile, metabolic, and collagenous proteins were not significantly different from the control level in SPR and ARG groups, while, surprisingly, in the SPI group all these parameters remained elevated. The discrepancy between LV weight reduction and maintenance of increased non-collagenous and collagenous protein concentration on the level of L-NAME group after spironolactone treatment suggests that spironolactone may have affected fluid volume in the left ventricle. It has been previously shown that prolonged arterial hypertension may not only be associated with fibrosis but also with an increase in interstitial fluid volume, namely two factors which cause deterioration of cardiac function (Laine *et al.* 1991). Indeed, L-NAME-induced LVH was shown to be associated with extracellular micro-edema as well as mitochondrial edema probably as a result of increased capillary permeability (Tribulová *et al.* 2000). In our previous work spontaneous regression of LVH resulted in the decrease of non-collagenous and collagenous protein concentration in the LV, but surprisingly, ACE-inhibitor captopril (similarly as spironolactone in this

experiment) maintained the enhanced level of all investigated proteins or even increased it, despite the complete reversion of the LVH (Bernátová *et al.* 2000). This finding may be related to the fact that ACE-inhibitors have diuretic effect as a consequence of their effect on tubular and glomerular functions (Romero *et al.* 1988). Analogically, also aldosterone receptors blockers have diuretic and natriuretic properties by antagonising the aldosterone effect on the level of renal distal tubules (Lijnen and Petrov 2000). We speculate that regression of LVH in the early phase of spironolactone-induced recovery is associated mainly with a significant reduction of the fluid volume in the myocardial tissue. It would need probably more time until spironolactone treatment would result also in the reduction of the concentration of non-collagenous and collagenous proteins.

In conclusion, spontaneous- and L-arginine-induced regression of hypertension resulted in regression of LVH and of protein remodeling of the LV. Spironolactone, however, despite normalization of the LV mass did not reverse contractile, metabolic and collagenous protein remodeling of the LV. We suggest that the early phase of spironolactone-induced recovery is associated with a significant reduction in myocardial fluid volume. Prolonging spironolactone treatment could presumably result also in the reduction of the non-collagenous and collagenous protein fraction concentration.

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Table 1 Body weight (BW), left ventricle weight (LVW), right ventricle weight (RVW) and relative right ventricle weight (RVW/BW).

	BW (g)	**LVW (mg)**	**RVW (mg)**	**RVW/BW (mg/g)**
CT4	376 ± 7	497.1 ± 12.2	158.3 ± 4.7	0.42 ± 0.02
LN4	333 ± 8*****	530.4 ± 13.8	143.7 ± 7.8	0.43 ± 0.02
CT7	403 ± 6	495.6 ± 17.5	181.0 ± 10	0.45 ± 0.02
SPR	373 ± 8†#	505.8 ± 19.6	189.2 ± 9.3†	0.51 ± 0.03
ARG	368 ± 8	485.6 ± 14.5	182.6 ± 9.0†	0.50 ± 0.03
SPI	362 ± 11#	446.4 ± 18.6†	161.1 ± 5.7	0.45 ± 0.02

CT4 and CT7, controls; LN4, 4-week L-NAME; SPR, 4-week L-NAME + 3-week placebo; ARG, 4-week L-NAME + 3-week L-arginine; SPI, 4-week L-NAME + 3-week spironolactone. *P<0.05 *vs.* CT4; #P<0.05 *vs.* CT7; †P<0.05 *vs.* LN4

Table 2 Content (mg) of individual proteins.

	Content (mg)			
	metabolic proteins	**contractile proteins**	**soluble collagenous proteins**	**insoluble collagenous proteins**
CT4	41.09 ± 1.02	44.10 ± 0.77	22.30 ± 0.082	5.89 ± 0.60
LN4	50.09 ± 2.28 *	52.03 ± 2.02 *	27.57 ± 1.08 *	9.63 ± 0.95 *
CT7	41.87 ± 1.47	44.04 ± 1.41	22.83 ± 1.27	6.17 ± 0.56
SPR	45.94 ± 2.28	49.94 ± 2.16	24.65 ± 0.92	7.18 ± 0.56
ARG	43.02 ± 1.74	46.71 ± 1.87	22.54 ± 0.70 †	5.99 ± 0.29 †
SPI	39.44 ± 1.98 †	42.23 ± 2.41 †	19.50 ± 0.56 †	6.03 ± 0.70 †

CT4 and CT7, controls; LN4, 4-week L-NAME; SPR, 4-week L-NAME + 3-week placebo; ARG, 4-week L-NAME + 3-week L-arginine; SPI, 4-week L-NAME + 3-week spironolactone. *P<0.05 *vs.* CT4; †P<0.05 *vs.* LN4

Figure captions

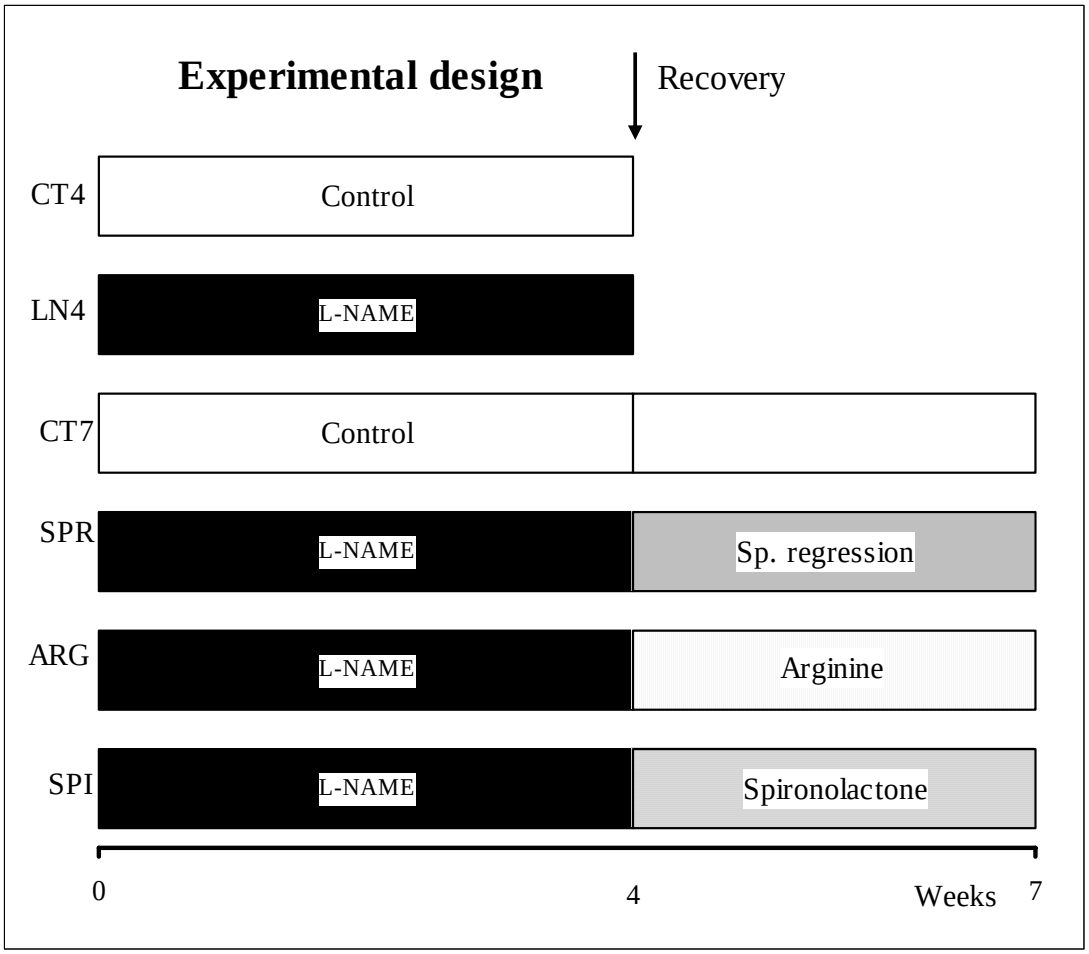
Figure 1. Experimental design. CT4 and CT7, controls; LN4, 4-week 40 mg/kg/day L-NAME; SPR, 4-week L-NAME + 3-week placebo; ARG, 4-week L-NAME + 3-week 1500 mg/kg/day L-arginine; SPI, 4-week L-NAME + 3-week 200 mg/kg/day spironolactone.

Figure 2. The effect of L-NAME-induced hypertension and its regression on left ventricle weight and the systolic blood pressure (SBP). CT4 and CT7, controls; LN4, 4-week L-NAME; SPR, 4-week L-NAME + 3-week placebo; ARG, 4-week L-NAME + 3-week L-arginine; SPI, 4-week L-NAME + 3-week spironolactone. ***P<0.001 *vs.* CT4; #P<0.05 *vs.* CT7; †P<0.05 *vs.* LN4.

Figure 3. Concentration (mg/g wet tissue) of metabolic and contractile proteins. CT4 and CT7, controls; LN4, 4-week L-NAME; SPR, 4-week L-NAME + 3-week placebo; ARG, 4-week L-NAME + 3-week L-arginine; SPI, 4-week L-NAME + 3-week spironolactone.*P<0.05 *vs.* CT4; #P<0.05 *vs.* CT7.

Figure 4. Concentration (mg/g wet tissue) of soluble and insoluble collagenous proteins. CT4 and CT7, controls; LN4, 4-week L-NAME; SPR, 4-week L-NAME + 3-week placebo; ARG, 4-week L-NAME + 3-week L-arginine; SPI, 4-week L-NAME + 3-week spironolactone. *P<0.05 *vs.* CT4; #P<0.05 *vs.* CT7; †P<0.05 *vs.* LN4.

Figure 1


Experimental design
Recovery
CT4
Control
LN4
L-NAME
CT7
Control
SPR
L-NAME
Sp. regression
ARG
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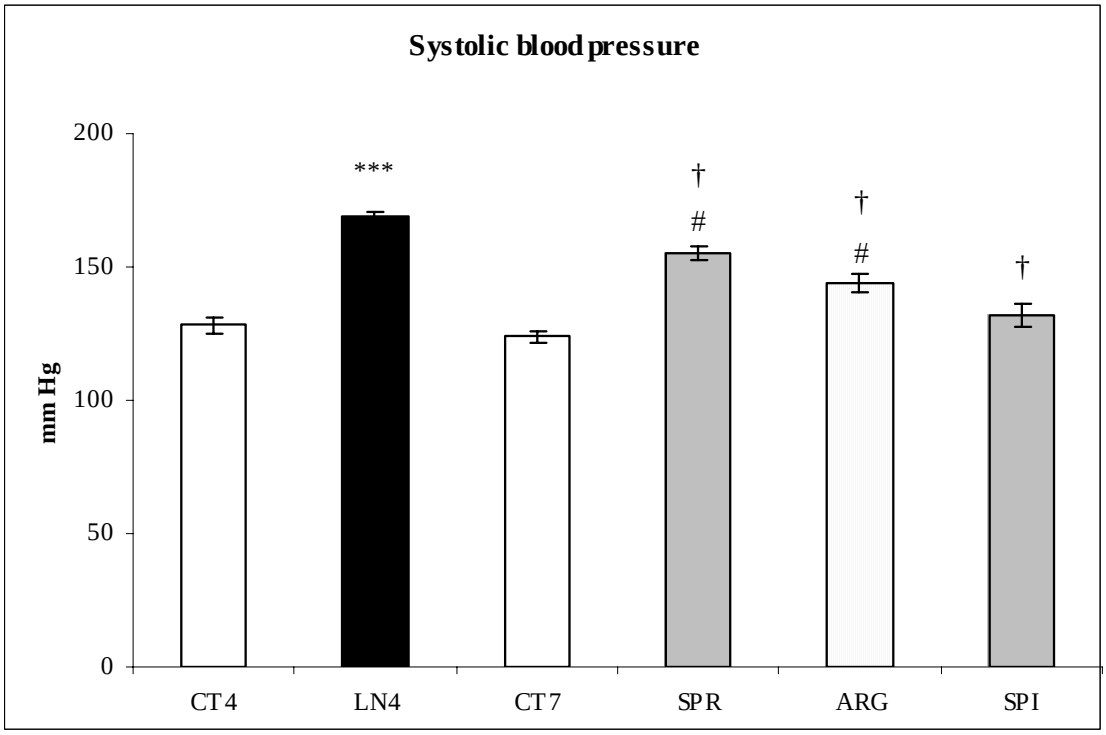
Arginine
SPI
L-NAME
Spironolactone
0
4
Weeks
7

Figure 2


Systolic blood pressure
mm Hg
200
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CT4
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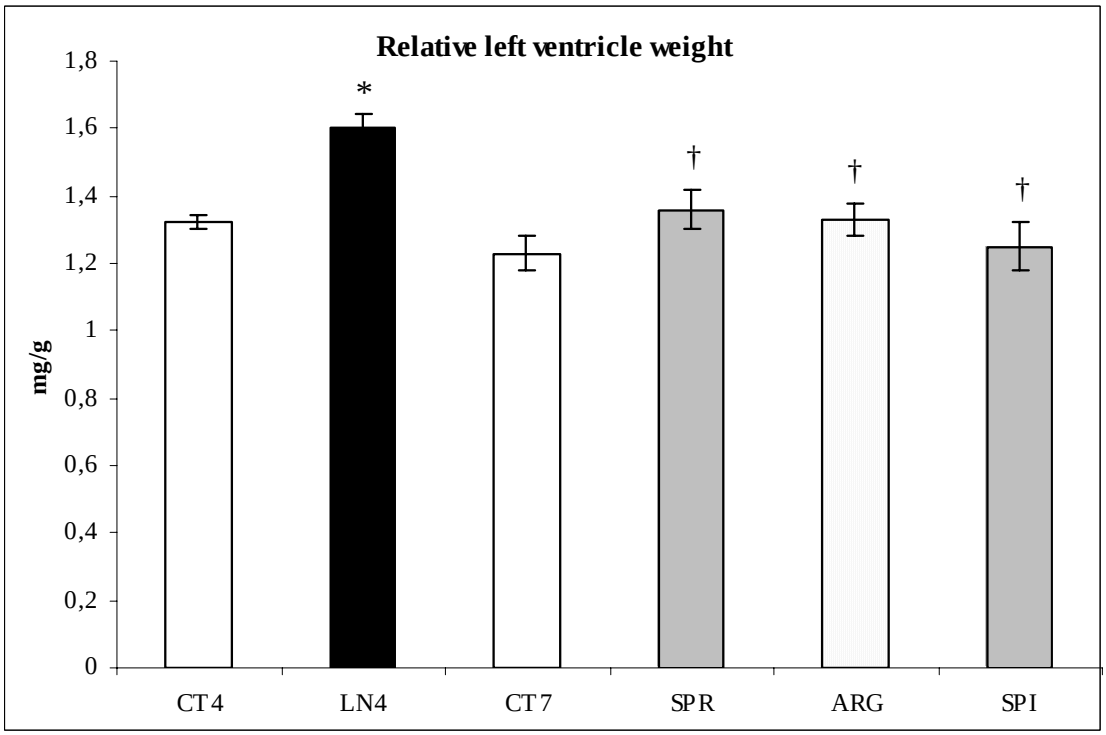

Relative left ventricle weight
mg/g
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
*
†
†
†
CT4
LN4
CT7
SPR
ARG
SPI

Figure 3

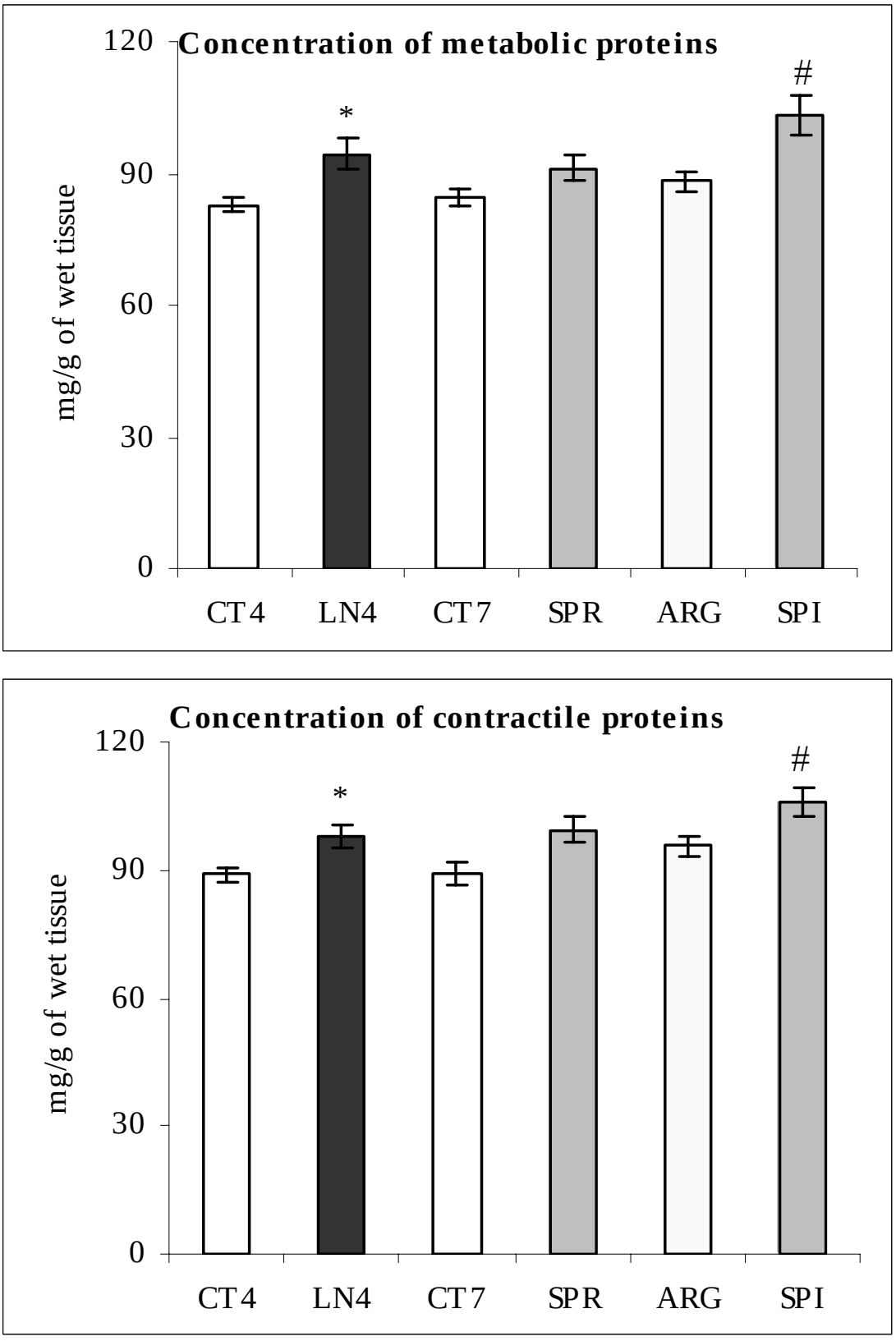
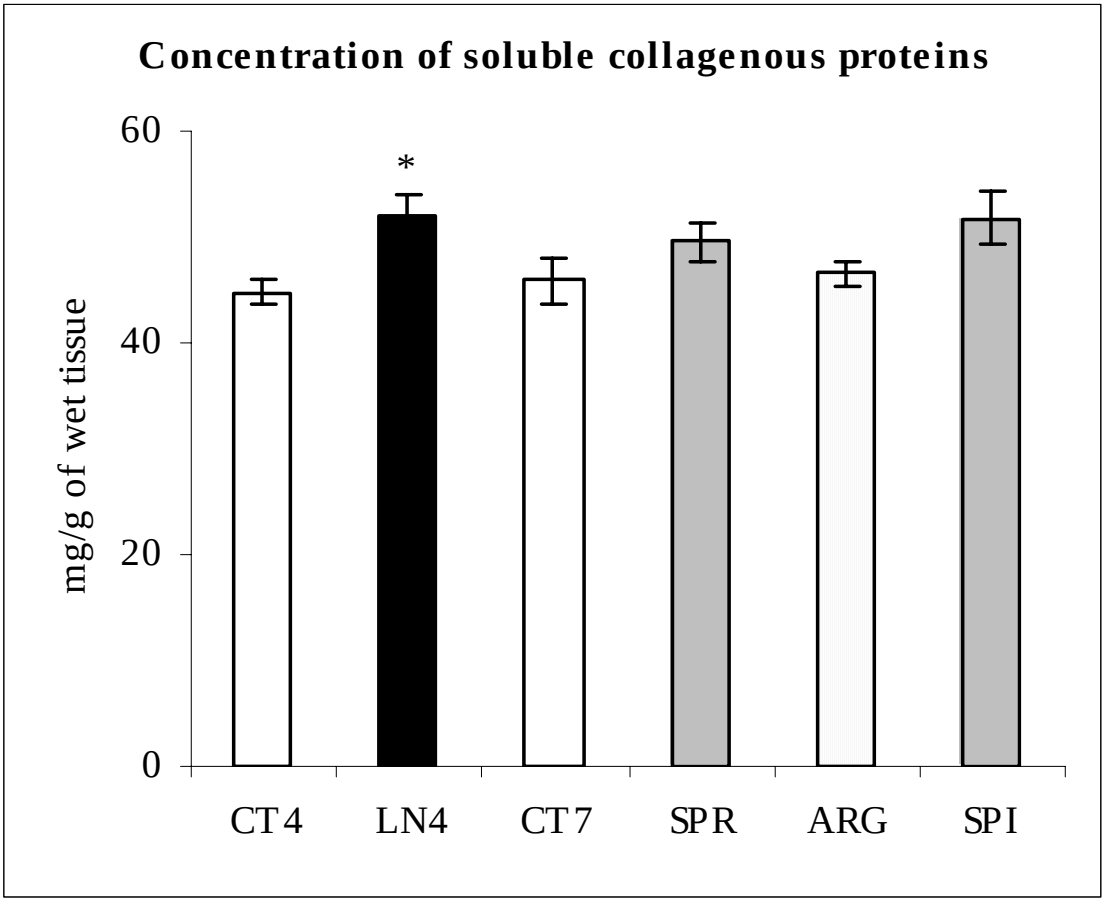
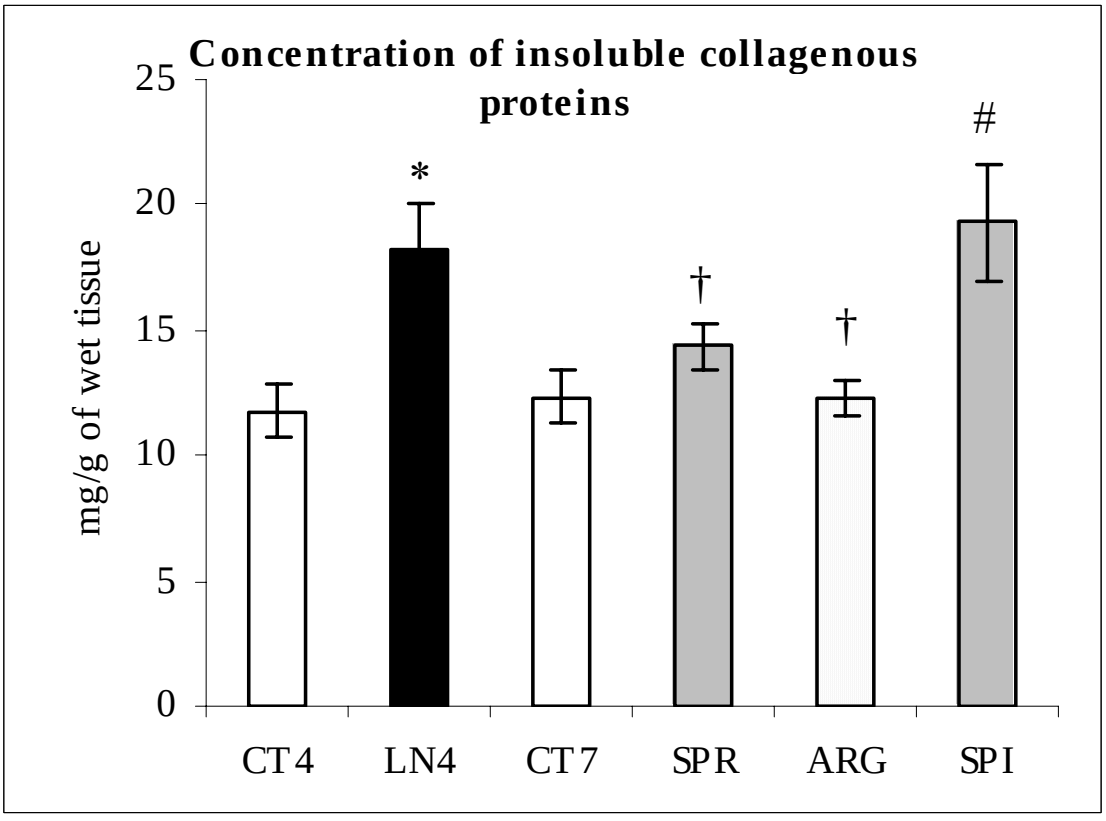

Concentration of metabolic proteins
mg/g of wet tissue
120
90
60
30
0
*
#
CT4
LN4
CT7
SPR
ARG
SPI
Concentration of contractile proteins
mg/g of wet tissue
120
90
60
30
0
*
#
CT4
LN4
CT7
SPR
ARG
SPI

Figure 4


Concentration of soluble collagenous proteins
mg/g of wet tissue
60
40
20
0
*
CT4
LN4
CT7
SPR
ARG
SPI


Concentration of insoluble collagenous proteins
mg/g of wet tissue
25
20
15
10
5
0
*
†
†
#
CT4
LN4
CT7
SPR
ARG
SPI