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RESEARCH PAPER

Effects of levosimendan on cardiac remodeling and cardiomyocyte apoptosis in hypertensive Dahl/Rapp rats

M Louhelainen[1], E Vahtola[1], P Kaheinen[2], H Leskinen[3], S Merasto[1], V Kytö[4], P Finckenberg[2], WS Colucci[5], J Levijoki[2], P Pollesello[2], H Haikala[2] and EMA Mervaala[1,6]

[1]*Institute of Biomedicine, Pharmacology, University of Helsinki, Helsinki, Finland;* [2]*Orion Pharma Ltd, Espoo, Finland;* [3]*Department of Pharmacology and Toxicology, University of Oulu, Oulu, Finland;* [4]*Department of Anatomy, University of Turku, Turku, Finland;* [5]*Boston University School of Medicine, Boston, MA, USA and* [6]*Department of Pharmacology and Toxicology, University of Kuopio, Kuopio, Finland*

Background and purpose: Progression of heart failure in hypertensive Dahl rats is associated with cardiac remodeling and increased cardiomyocyte apoptosis. This study was conducted to study whether treatment with a novel inotropic vasodilator compound, levosimendan, could prevent hypertension-induced cardiac remodeling and cardiomyocyte apoptosis.
Experimental approach: 6-week-old salt-sensitive Dahl/Rapp rats received levosimendan (0.3 mg kg^{-1} and 3 mg kg^{-1} via drinking fluid) and high salt diet (NaCl 7%) for 7 weeks, Dahl/Rapp rats on low-salt diet served as controls. Blood pressure, cardiac functions by echocardiography, cardiomyocyte apoptosis by TUNEL technique, tissue morphology, myocardial expression of calcium cycling proteins, and markers of neurohumoral activation were determined.
Key Results: Untreated Dahl/Rapp rats on high salt diet developed severe hypertension, cardiac hypertrophy and moderate systolic dysfunction. 38% of Dahl/Rapp rats (9/24) survived the 7-week-follow-up period. Cardiomyocyte apoptosis was increased by 6-fold during high salt diet. Levosimendan improved survival (survival rates in low- and high-dose levosimendan groups 12/12 and 9/12, $p<0.001$ and $p=0.05$, respectively), increased cardiac function, and ameliorated cardiac hypertrophy. Levosimendan dose-dependently prevented cardiomyocyte apoptosis. Levosimendan normalized salt-induced increased expression of natriuretic peptide, and decreased urinary noradrenaline excretion. Levosimendan also corrected salt-induced decreases in myocardial SERCA2a protein expression and myocardial SERCA2a/NCX-ratio.
Conclusions and Implications: Improved survival by the novel inotropic vasodilator levosimendan in hypertensive Dahl/Rapp rats is mediated, at least in part, by amelioration of hypertension-induced cardiac remodeling and cardiomyocyte apoptosis.
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Keywords: inotropic agents; remodeling; hypertension; calcium (cellular); apoptosis

Abbreviations: ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; K_{ATP}, ATP-dependent K^+ channel; LC-MS/MS, liquid chromatography-tandem mass spectrometry; NCX, Na^+–Ca^{2+} exchanger; PKCε, protein kinase C epsilon; PRA, plasma renin activity; ROS, reactive oxygen species; SERCA2a, sarco/endoplasmic reticulum Ca^{2+}-ATPase 2a; TUNEL, terminal deoxynucleotidyl transferase labeling

Introduction

Congestive heart failure is a complex syndrome, which includes disturbances in ventricular function, neurohumoral and cytokine activation and increased arrhythmias (Hasenfuss and Pieske, 2002; Jessup and Brozena, 2003). Accumulating evidence indicates a central role for myocyte mishandling of calcium in the pathogenesis of heart failure (del Monte and Hajjar, 2003). Defects in various steps of cardiac excitation–contraction coupling have been identified in both human and experimental models of heart failure, characterized as prolonged calcium transients with elevated end-diastolic and decreased systolic intracellular calcium concentrations and prolonged relaxation phases (Narula *et al.*, 1996). Emerging evidence also suggests that myocyte loss owing to apoptosis plays an important pathophysiological role in the progression of myocardial dysfunction (Narula *et al.*, 1996; Olivetti *et al.*, 1997; Kang and Izumo, 2000).

Correspondence: Professor E Mervaala, Department of Pharmacology and Toxicology, University of Kuopio, PO Box 1627, FIN-70211 Kuopio, Finland.
E-mail: eero.mervaala@uku.fi
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Levosimendan is a novel inotropic agent used in the management of acute decompensated heart failure that mediates its cardiac effect mainly by the calcium sensitization of the contractile protein troponin C (Innes and Wagstaff, 2003). Levosimendan binds to the calcium-saturated N-terminal domain of troponin C in cardiac muscle and stabilizes the troponin molecule with subsequent prolongation of its effect on the contractile proteins (Haikala *et al*., 1995; Innes and Wagstaff, 2003). Besides increasing the strength of cardiac contractions, levosimendan also exerts vasodilatory effects through opening of the ATP-dependent K^+ channels (K_{ATP}) (Kaheinen *et al*., 2001). Although levosimendan displays some structural similarities with phosphodiesterase inhibitors, it is believed that the inotropic action of levosimendan is due primarily to calcium sensitization (Haikala *et al*., 1995; Innes and Wagstaff, 2003). Previous clinical studies have provided evidence that short-term infusion of levosimendan improves the hemodynamic function and may improve long-term survival in patients with decompensated heart failure (Slawsky *et al*., 2000; Follath *et al*., 2002; Kivikko *et al*., 2003). It has also been suggested that levosimendan may exert antiapoptotic properties that are linked to activation of mitochondrial K_{ATP} channels (Parissis *et al*., 2004; Maytin and Colucci, 2005).

The inbred Dahl/Rapp rat strain provides a widely used animal model for salt-sensitive hypertension and hypertension-induced target organ damage (Rapp and Dene, 1985; Walder *et al*., 1996). Salt-sensitive Dahl/Rapp rats rapidly and uniformly develop fulminant hypertension and begin to die after only 3 weeks on high-salt diet, and the overall mortality is usually almost 100% when kept on high-salt diet for 8 weeks (Rapp and Dene, 1985; Walder *et al*., 1996). Dahl/Rapp rats develop severe target-organ damage such as cardiac hypertrophy, heart failure, as well as vascular and renal damage (Rapp and Dene, 1985; Walder *et al*., 1996). Previous studies have revealed only relatively modest alterations in myocardial calcium homeostasis during the transition from compensated left ventricular hypertrophy to heart failure (Kihara and Sasayama, 1997; Yoneda *et al*., 2001). Noguchi *et al*. (2003) identified a thin-filament defect that causes a reduction in calcium sensitivity in the failing hearts of Dahl salt-sensitive rats. Furthermore, Ikeda *et al*. (2002) reported increased cardiomyocyte apoptosis at the stage of heart failure in Dahl salt-sensitive rats. The aim of the present study was to investigate whether oral treatment with the novel inotropic vasodilator levosimendan could prevent hypertension-induced cardiac remodeling and salt-induced mortality in Dahl/Rapp rats. The influence of levosimendan on cardiomyocyte apoptosis, neurohumoral activation and myocardial expression of calcium cycling proteins were also examined.

Materials and methods

Experimental animals, dietary and drug regimens and sample preparation

Sixty-six 6-week-old male Dahl/Rapp salt-sensitive rats (SS/JrHsd) were purchased from Harlan (Indianapolis, IN, USA). Development and characteristics of this inbred strain of Dahl salt-sensitive rat has been described in detail previously (Rapp and Dene, 1985; Walder *et al*., 1996). The protocols were approved by the Animal Experimentation Committee of the University of Helsinki, Finland, whose standards correspond to those of the American Physiological Society. Dahl/Rapp rats were divided into four groups to receive the following diet and drug regimens for 7 weeks: (1) Dahl/Rapp rats on high-salt diet ($n=36$), (2) Dahl/Rapp rats on high-salt diet + low-dose levosimendan ($0.3\,mg\,kg^{-1}$) ($n=12$), (3) Dahl/Rapp rats on high-salt diet + high-dose levosimendan ($mg\,kg^{-1}$) ($n=12$), and (4) Dahl/Rapp rats on low-salt diet ($n=6$). To examine the influence of levosimendan during the late-stage of hypertension, an additional study group ($n=12$) was treated with levosimendan ($mg\,kg^{-1}$) from week 4 to week 7 for observational reasons only. In an additional pharmacokinetic experiment the diurnal blood drug concentrations of levosimendan and its long-lasting metabolite (OR-1896) was examined after 2 weeks oral treatment with low- and high-dose levosimendan ($n=4$ in both groups). High-salt diet was produced by adding NaCl (Riedel-de-Haen) to commercial low-salt diet (Na 0.3%, K 0.8%, Mg 0.2%; Harlan). Levosimendan (the (−)enantiomer of {[4-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)phenyl]-hydrazono}-propanedinitrile) (Orion Pharma, Finland) was given via drinking fluid at concentrations (1 and $10\,mg\,l^{-1}$) that have produced beneficial effects in previous rat experiments (Levijoki *et al*., 2001). Fresh levosimendan solutions were prepared daily. The rats had free access to chow and drinking water. Systolic blood pressure was measured by using a tail cuff blood pressure analyzer (Apollo-2AB Blood Pressure Analyzer, Model 179-2AB, IITC Life Science, Woodland Hills, CA, USA) at weeks 3.5 and 7 by the same technician. Urine was collected over 2 consecutive days in metabolic cages for albumin and catecholamine measurements. Urine volumes and water intakes were measured gravimetrically. Rats were then anesthetized with CO_2/O_2 (AGA, Riihimäki, Finland) and decapitated. Blood samples were collected for plasma brain natriuretic peptide (BNP), aldosterone and levosimendan measurements using ethylenediaminetetraacetic acid as anticoagulant. The heart and kidneys were excised, washed with ice-cold saline, blotted dry and weighed. Tissue samples were snap-frozen in liquid nitrogen. All samples were stored at −80°C until assayed. Samples for conventional morphology were fixed with 10% formalin and processed in paraffin with routine techniques.

Echocardiography

Transthoracic echocardiography was performed using a Toshiba Ultrasound System and a 15-MHz linear transducer under light isoflurane anesthesia on all surviving rats at the end of the experimental period. Using two-dimensional imaging (Gibson method), a short-axis view of the left ventricle at the level of the papillary muscles was obtained and the two dimensionally guided M-mode recording through the anterior and posterior walls (PWs) of the left ventricle was obtained. Left ventricle (LV) end-systolic (LVESD) and end-diastolic (LVEDD) dimensions as well as interventricular septum (IVS) and PW thickness were measured from the M-mode tracings. LV shortening fraction

(LVFS) and ejection fraction (EF), measures of LV systolic function, were calculated from the M-mode LV dimensions using the following equations:

$$\begin{aligned}\text{LVFS}(\%) &= \{(\text{LVEDD} - \text{LVESD})/\text{LVEDD}\} \times 100\\ \text{EF} &= \text{SV}/\text{EDV}\\ \text{SV} &= \text{EDV} - \text{ESV}\\ \text{EDV} &= 0.52 \times (0.98 \times (\text{LVIDD}/10) + 5.90) \times (\text{LVIDD}/10)^2\\ \text{ESV} &= 0.52 \times (1.14 \times (\text{LVIDS}/10) + 4.18) \times (\text{LVIDS}/10)^2\end{aligned}$$

LVIDD is the diameter of the short-axis left ventricle in end diastole and LVIDS is the diameter of the short-axis left ventricle in end systole.

Heart morphology and immunohistochemistry

Histology of the myocardial samples was evaluated without the knowledge of the treatments, with conventional light microscopy. The severity of observed lesions was graded with numerical values denoting to the degree of damage at the whole tissue level. The following system of severity grading was used: '(0, no abnormalities detected) 1, minimal; 2, mild; 3, moderate; 4, marked; or 5, severe' (Herbert *et al*., 2002). In the cardiac samples, severity grading was performed on the coronary arteries and the myocardium. The observed lesions in the coronary arteries ranged from minimally thickened media and slight increase of connective tissue around the arteries to severe hyperplasia of intimal/medial layer and fibrinoid necrosis of the arterial wall with adventitial scarring and perivascular inflammation. Lesions in the myocardium ranged from a focal increase of slender connective tissue bundles to severe necrosis of the myocardium with inflammatory infiltrate and presence of older myocardial scars.

Perivascular monocyte/macrophage infiltration was examined by immunohistochemistry using rat ED-1 (Serotec Ltd, Oslo, Norway) as primary antibody, and cardiac nicotinamide adenine dinucleotide phosphate (NADPH) oxidase expression using p47phox (BD Biosciences, Pharmingen, USA) as primary antibody.

Cardiomyocyte apoptosis

Cardiomyocyte apoptosis was assessed by the terminal deoxynucleotide transferase mediated ddUTP nick end labeling (TUNEL) assay as described previously (Kytö *et al*., 2004). In brief, nuclear DNA strand breaks were end-labeled with digoxigenin-conjugated dideoxy-UTP by terminal transferase and visualized immunohistochemically with digoxigenin antibody conjugated to alkaline phosphatase. The assay was standardized with the use of adjacent tissue sections treated with DNase I to induce DNA fragmentation as a positive control for apoptosis. The percentages of TUNEL-positive cardiomyocytes were calculated in transverse sections of tissue from the left ventricle, using light microscopy ($\times 200$ magnification) with an ocular grid. An average of 190 fields each containing on average 160 myocyte nuclei were studied. Cardiomyocytes were identified by the presence of myofilaments surrounding the nucleus.

Biochemical determinations

Plasma BNP (BNP-45, Peninsula Laboratories, USA), plasma renin activity (PRA) (Angiotensin I RIA kit, Diasorin, Italy), and aldosterone (Coat-a-Count Aldosterone RIA kit, DPC, USA) were determined by RIA according to the manufacturer's instructions. Plasma samples were analyzed for levosimendan and OR-1896 by liquid chromatography–tandem mass spectrometry (LC-MS/MS) (Kivikko *et al*., 2003). Urinary albumin was measured by ELISA using rat albumin as a standard (Celltrend, Luckenwalde, Germany). Urinary noradrenaline was analyzed using the isocratic ion-pair reversed-phase high-performance liquid chromatography method with electrochemical detection.

Myocardial gene expression analysis by quantitative real-time reverse transcriptase PCR assay (RT-PCR)

The gene expressions of atrial natriuretic peptide (ANP), BNP, sarco/endoplasmic reticulum Ca^{2+}–ATPase 2a (SERCA2), Na^{+}–Ca^{2+} exchanger (NCX), osteopontin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were determined by quantitative real-time RT-PCR (LightCycler, Roche Diagnostics, Neuilly Sur Seine, France). The following primers were used: ANP forward CCGATAGATTCTGCCCTCTT GAA, reverse CCCGAAGCAGCTTGATCTTC; BNP forward AA CTTCTAAAAAGACTCCTTAGGTCTCAA, reverse GCCATCTTG CAATTTCGAAGTC; SERCA2 forward TTCCGTTACCTGGC TATT, reverse CATCGGATACGGGGAC; NCX forward ATGCTT CGACTAAGTCTCCCA, reverse ACAAAATACACAGTTGCTC TAG; GAPDH forward GGATGCAGGGATGATGTTCT, reverse GAAGGGCTCATGACCACAGT; osteopontin forward CCAG CACACAAGCAGACGTT, reverse TCAGTCCATAAGCCAAGCT ATCAC. The quantities of ANP, BNP, SERCA2, NCX, osteopontin and GAPDH PCR products were quantified with an external standard curve amplified from purified PCR product.

Myocardial SERCA2 and NCX expressions by Western blot

Western blot analysis was performed by standard procedures. Myocardial samples (15 μg protein per lane) were electrophoretically separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (8% acrylamide). The proteins were transferred onto a polyvinylidene fluoride membrane (Immobilon, Millipore, Bedford, MA, USA) and the membranes were then blocked overnight, at $+4^{\circ}$C, in 5% milk powder–TBS−0.01% Tween solution. The membrane was washed and probed for 1 h at room temperature with the primary antibody (rabbit anti-NCX, 1:5000 AD). After washing, the membrane was probed with peroxidase-conjugated secondary antibody (anti-rabbit 1:5000; Chemicon). Detection was accomplished with an enhanced chemiluminescence kit (Advanced ECL, Amersham Biotech, Buckinghamshire, UK) and the blots were exposed to X-ray film (Hyperfilm-ECL; Amersham Pharmacia Biotech). The membrane was stripped from antibodies (Stripping Buffer; Pierce) and after washing it was reprobed with a second antibody (rabbit anti-Serca2, 1:5000 Abcam), probing with secondary antibody and detection were carried out as described above. The films were scanned in a densitometer (Syngene, Cambridge, UK) and a semi-quantitative measurement of the

relative intensity of each protein band was performed using the 'GeneSnap'-software program (Syngene).

Statistical analysis

Data are presented as means ± s.e.m. Statistically significant differences in mean values were tested by ANOVA and Tukey's test. The differences were considered significant when $P<0.05$. The Kaplan–Meier test was used for survival analysis. The data were analyzed using SPSS statistical software (SPSS Inc., USA).

Results

Survival rates in Dahl/Rapp salt-sensitive rats

Only 38% of Dahl/Rapp rats on high-salt diet (9/24) survived the 7-week follow-up period (Figure 1a), significantly lower than the survival rate in Dahl/Rapp rats on low-salt controls. Levosimendan treatment when started in the early stages of hypertension, improved survival in Dahl/Rapp rats at both doses. None of the rats in the low-dose levosimendan group died and only three out of 12 rats died in the high-dose levosimendan group, yielding survival rates greater than that in Dahl/Rapp rats on high-salt diet. Interestingly, late-stage levosimendan treatment ($3\,mg\,kg^{-1}$ from weeks 4 to 7) also improved survival in Dahl/Rapp rats (Figure 1b). Untreated Dahl/Rapp rats on high-salt diet gained weight less compared to levosimendan-treated Dahl/Rapp rats on high-salt diet and to Dahl/Rapp rats low-salt controls (data not shown).

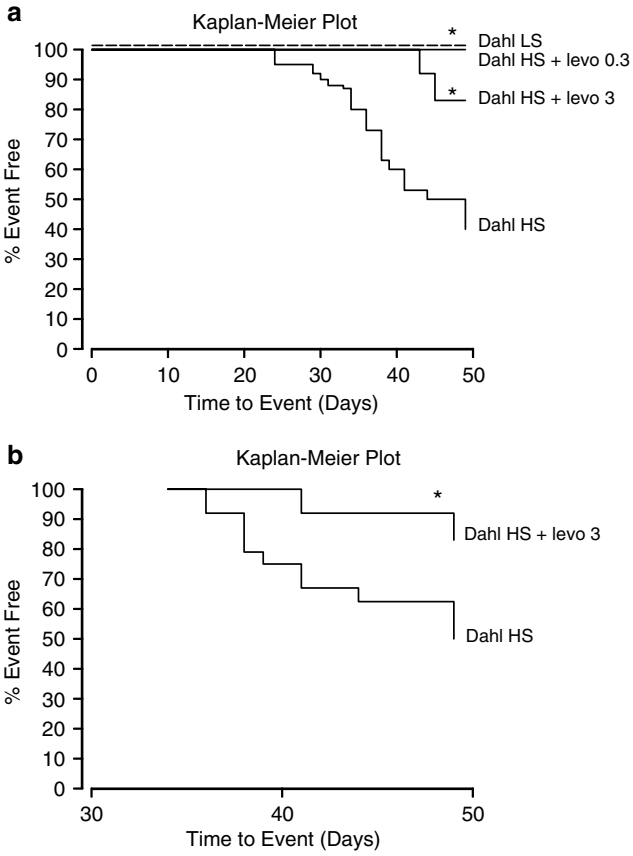

Figure 1 Survival curves of Dahl/Rapp rats on high salt diet treated with levosimendan at two different doses compared to those for untreated Dahl/Rapp rats on high-salt diet and Dahl/Rapp rats on low-salt diet. Levosimendan was given either during the whole 7-week-follow-up period (**a**) or only during the late stage of hypertension, from week 4 to week 7 (**b**). The concentration of levosimendan in drinking water (given *ad libitum*) was $1\,mg\,l^{-1}$ (low dose) or $10\,mg\,l^{-1}$ (high dose) corresponding to daily doses of $0.3–3\,mg\,kg^{-1}$, respectively. The log rank test was used to compare the Kaplan–Meier survival curves to each other. * denotes $P<0.05$ compared to Dahl/Rapp rats on high-salt diet.

Blood pressure, cardiac hypertrophy and cardiac morphology

Dahl/Rapp rats on high-salt diet developed pronounced hypertension (Figure 2a and b) with cardiac hypertrophy (Figure 3a), coronary damage (Figure 3b) and to a lesser extent myocardial damage (Figure 3c). This group of animals showed intimal hyperplasia, fibrinoid necrosis of the arteries with marked medial thickening and adventitial scarring, evident myocardial infarcts with inflammation (Figure 3d), and perivascular monocyte/macrophage infiltration (Figure 3e). The expression of reactive oxygen species (ROS)-generating NADPH oxidase, assessed by p47phox immunohistochemistry, was found both in perivascular space and in cardiomyocytes (Figure 3f).

High-dose levosimendan produced a transient decrease in blood pressure, whereas low-dose levosimendan did not influence blood pressure in Dahl/Rapp rats (Figure 2a and b). Levosimendan did not influence heart rate (Figure 2c and d). Both levosimendan doses equally prevent the development of cardiac hypertrophy when measured as heart weight-to-body weight ratio (Figure 3a). However, levosimendan treatments did not significantly decrease coronary or myocardial damage scores (Figure 3b and c).

Echocardiography

Dahl/Rapp rats on high-salt diet showed deterioration of cardiac functions and left ventricular hypertrophy when assessed by echocardiography. Ejection fraction and fractional shortening were decreased in Dahl/Rapp rats on high-salt diet (Table 1). Levosimendan dose-dependently improved EF and fractional shortening. Left ventricular diameter in systole was markedly decreased by levosimendan.

Cardiomyocyte apoptosis

In Dahl/Rapp rats on high-salt diet, cardiomyocyte apoptosis detected by TUNEL technique was increased by sixfold as compared to low-salt controls (Figure 4). Levosimendan dose-dependently decreased the number of apoptotic cardiomyocytes in Dahl/Rapp rats on high-salt diet.

Myocardial SERCA2 and NCX protein and mRNA expressions

In Dahl/Rapp rats on high-salt diet, myocardial SERCA2 expression was decreased by 40%, NCX expression unaltered and the SERCA2-to-NCX-ratio decreased as compared to

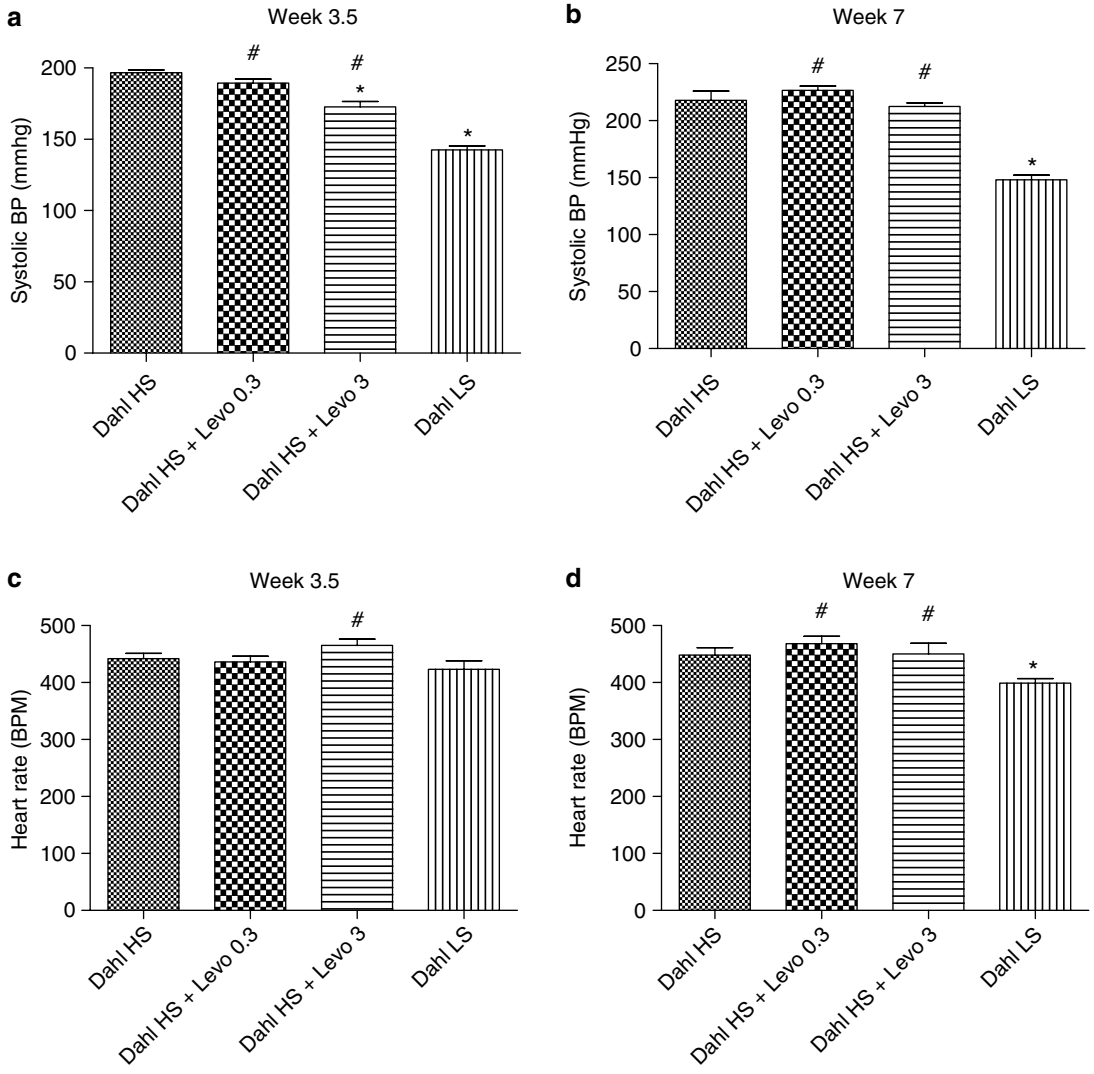

Figure 2 Bar graphs showing the effects of levosimendan treatment on systolic blood pressure (**a** and **b**) and heart rate (**c** and **d**) in Dahl/Rapp rats on high-salt diet. In the figure, Dahl HS denotes untreated Dahl/Rapp rats on high-salt diet; Dahl HS + LS 0.3 denotes Dahl/Rapp rats treated with low-dose levosimendan; Dahl HS + LS 3 denotes Dahl/Rapp rats treated with high-dose levosimendan; Dahl LS denotes Dahl/Rapp controls on low-salt diet. High-dose levosimendan produced a transient decrease in blood pressure (week 3.5), whereas low-dose levosimendan did not influence blood pressure or heart rate. Means ± s.e.m. are given, $n = 6$–12 in each group. * denotes $P < 0.05$ compared to Dahl/Rapp rats on high-salt diet; # denotes $P < 0.05$ compared to Dahl/Rapp rats on low-salt diet.

Dahl/Rapp controls on low-salt diet (Figure 5a–c). Both levosimendan doses prevented the decrease in SERCA2 expression as well as in the SERCA2-to-NCX-ratio in the heart.

A similar trend was also found in SERCA2 and NCX mRNA expressions in the heart. However, the difference between high- and low-salt diet groups did not quite reach statistical significance, except in SERCA2-to-NCX mRNA ratio (Figure 5d–f).

Myocardial ANP, BNP and osteopontin mRNA expressions

Myocardial ANP mRNA expression (Figure 6a) was increased by fivefold in Dahl/Rapp rats on high-salt diet and myocardial BNP mRNA expression nonsignificantly, by 50% (Figure 6b). High-dose levosimendan prevented the increase in ANP mRNA expression keeping it at the level found in the group of Dahl/Rapp rats maintained on low-salt diet.

High-dose levosimendan prevented the salt-induced increase in myocardial osteopontin mRNA expression in Dahl/Rapp rats (Figure 6c).

Biochemical and hormonal analyses

Dahl/Rapp rats on high-salt diet developed pronounced renal damage when assessed by 24-h urinary albumin excretion (Table 2). They also showed increased plasma BNP concentration as compared to Dahl/Rapp controls on low-salt diet. There was no difference in PRA or serum aldosterone level. Levosimendan did not influence albuminuria, but it decreased plasma BNP to levels found in Dahl/Rapp rats low-salt controls, and decreased urinary noradre-

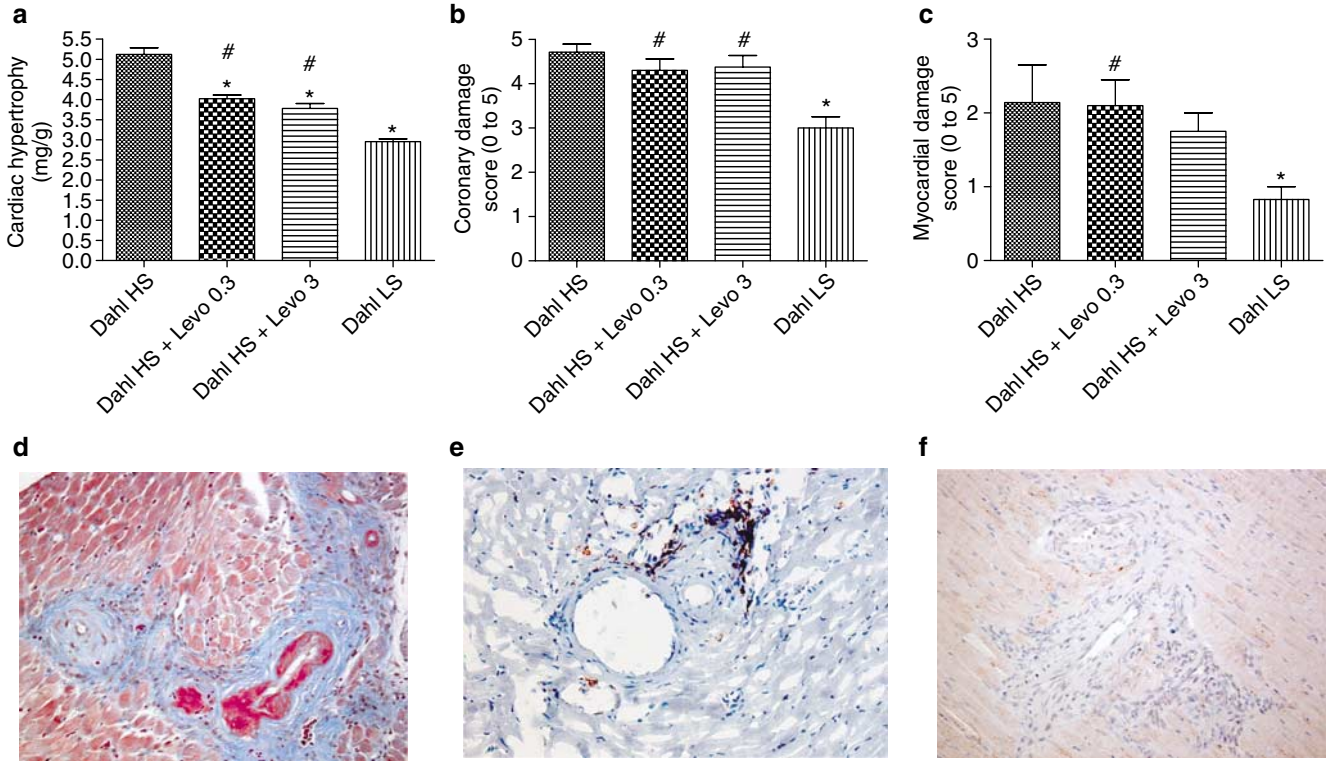

Figure 3 Bar graphs showing the effects of the 7-week levosimendan treatment on cardiac hypertrophy measured as heart weight-to-body weight ratio (**a**), coronary damage score (**b**) and myocardial damage score (**c**) in Dahl/Rapp rats on high-salt diet. Representative photomicrograph from the heart of Dahl/Rapp rat on high-salt diet is shown in (**d**), perivascular monocyte/macrophage infiltration in (**e**), and myocardial p47phox expression in (**f**). For abbreviations, see Figure 2. Means ± s.e.m. are given, $n = 6$–12 in each group. * denotes $P < 0.05$ compared to Dahl SS rats on high-salt diet; # denotes $P < 0.05$ compared to Dahl/Rapp rats on low-salt diet.

Table 1 Echocardiographic parameters in Dahl/Rapp salt-sensitive rats

Variable	*Dahl/Rapp on high-salt diet*	*Dahl/Rapp on high-salt diet + Levo 0.3*	*Dahl/Rapp on high-salt diet + Levo 3*	*Dahl/Rapp on low-salt diet*	*ANOVA* P-*value*
IVS (d), mm	2.54 ± 0.04	2.20 ± 0.06*	2.26 ± 0.10*	1.92 ± 0.08*	< 0.001
LVD (d), mm	7.42 ± 0.08	6.86 ± 0.14* #¤	7.67 ± 0.14	8.21 ± 0.11*	< 0.001
LVPW (d), mm	2.45 ± 0.04	2.29 ± 0.09#	2.13 ± 0.06*	1.88 ± 0.08*	< 0.001
EF, %	83.0 ± 0.6	92.7 ± 0.7*#	95.1 ± 0.5*#	87.2 ± 0.8*	< 0.001
FS, %	50.9 ± 0.8	68.0 ± 1.7*#	73.2 ± 1.2*#	56.9 ± 1.2	< 0.001

Abbreviations: EF, ejection fraction; IVS, interventricular septum; LVD, left ventricle diastolic; LVPW, left ventricle posterior wall.
Four experimental groups of animals were used: Dahl/Rapp rats on high-salt diet with and without levosimendan (Levo, 0.3 or 3 mg kg^{-1} via drinking fluid for 7 weeks) and Dahl/Rapp controls receiving low-salt diet. Data shown are means ± s.e.m., $n = 6$–12 in each group. *$P < 0.05$ compared to Dahl/Rapp rats on high-salt diet; #$P < 0.05$ compared to Dahl/Rapp rats on low-salt diet, ¤$P < 0.05$ compared to Dahl/Rapp rats on high-salt diet + Levo 3.

naline excretion (Table 2). PRA or serum aldosterone was not influenced by levosimendan.

During the 7-week-experiment period the daily dosages of low- and high-dose levosimendan treatments averaged 0.36 ± 0.01 and 3.6 ± 0.1 mg kg^{-1}, respectively (Figure 7a). Plasma levosimendan concentration exhibited typical diurnal variation with highest plasma concentrations during night when the rats are active (Figure 7b). The mean 24-h plasma concentrations of levosimendan and its active metabolite OR-1896 (Figure 7c) in Dahl/Rapp rats receiving the low-dose levosimendan treatment were 6.4 ± 1.3 and 4.5 ± 0.2 ng ml^{-1}, and the corresponding drug concentrations in the high-dose levosimendan group were 79 ± 19 and 42 ± 15 ng ml^{-1}, respectively. Plasma OR-1896 level did not correlate with BNP concentration (ANOVA $P = 0.334$).

Discussion

In the present study, the effects of oral levosimendan on cardiac remodeling and cardiomyocyte apoptosis were examined in Dahl/Rapp salt-sensitive rats with fulminant hypertension and severe target-organ damage. The important finding of our study was that levosimendan improved

the survival in Dahl/Rapp rats on high-salt diet without major changes in systemic blood pressure or heart rate. Levosimendan improved systolic function, prevented cardiac remodeling and dose-dependently decreased the number of apoptotic cardiomyocytes.

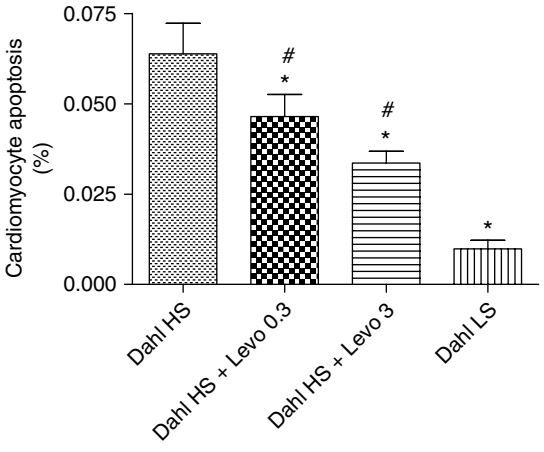

Figure 4 Bar graphs showing the effects of the 7-week levosimendan treatment on cardiomyocyte apoptosis in Dahl/Rapp rats on high-salt diet. For abbreviations, see Figure 2. Means ± s.e.m. are given, $n = 6$–12 in each group. * denotes $P < 0.05$ compared to Dahl/Rapp rats on high-salt diet; # denotes $P < 0.05$ compared to Dahl/Rapp rats on low-salt diet.

In the present study, both low and high levosimendan doses improved survival in Dahl/Rapp rats without significantly influencing systolic blood pressure, heart rate, or albuminuria suggesting that the beneficial effect of levosimendan on salt-induced mortality was mediated, to a great extent, via mechanisms independent of systemic blood pressure or renal function. The finding that late levosimendan treatment, which was started when the rats already have developed manifest hypertension, also improved survival, supports this notion. In rats, levosimendan is absorbed rapidly and extensively (Orion Pharma, unpublished data). Whereas levosimendan has a relatively short elimination half-life in rats (0.76 h), the active metabolite OR-1896 is long-lasting ($T\frac{1}{2}$ 6.5 h) (Orion Pharma, unpublished data). At therapeutic concentrations the primary mechanism of action of levosimendan is calcium sensitization of the contractile protein troponin C (Haikala *et al*., 1995; Innes and Wagstaff, 2003). Consistently we noticed a significant dose-dependent improvement in cardiac function by oral levosimendan. The present study also confirmed the previous finding by Klotz *et al*. (2006) that development of heart failure in hypertensive Dahl rats is associated with a relatively well-preserved ejection fraction.

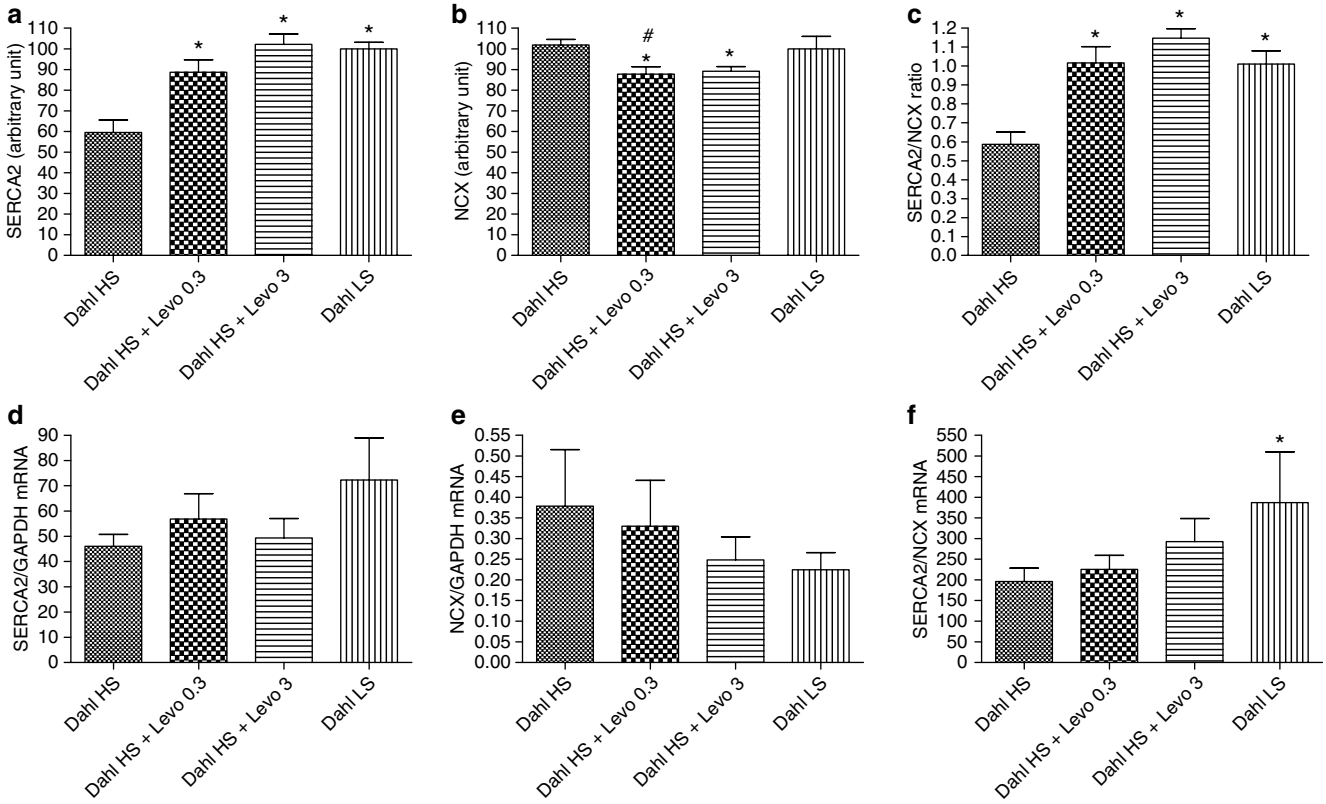

Figure 5 Bar graphs showing the effects of the 7-week levosimendan treatment on myocardial SERCA2 protein (**a**) and mRNA (**d**) expression, NCX protein (**b**) and mRNA (**e**) expression and SERCA2-to-NCX-protein (**c**) and mRNA (**f**) ratios in Dahl/Rapp rats on high-salt diet. For abbreviations, see Figure 2. Means ± s.e.m. are given, $n = 6$–12 in each group. * denotes $P < 0.05$ compared to Dahl/Rapp rats on high-salt diet; # denotes $P < 0.05$ compared to Dahl/Rapp rats on low-salt diet.

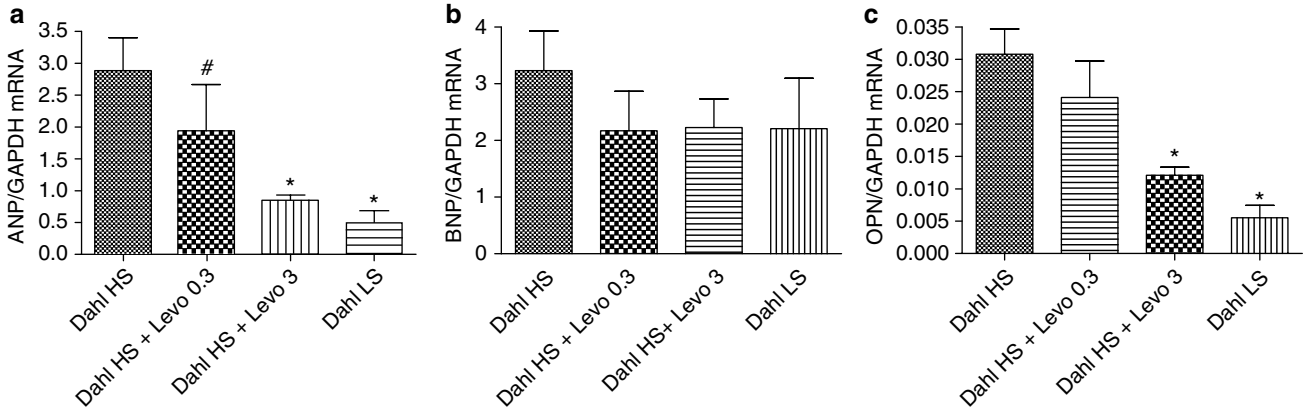

Figure 6 Bar graphs showing the effects of the 7-week levosimendan treatment on myocardial ANP (**a**), BNP (**b**) and osteopontin mRNA expressions (**c**) in Dahl/Rapp rats on high-salt diet. For abbreviations, see Figure 2. Means ± s.e.m. are given, $n = 6$–12 in each group. * denotes $P < 0.05$ compared to Dahl/Rapp rats on high-salt diet.

Table 2 Effects of levosimendan on some biochemical and hormonal markers of neurohumoral activation in Dahl/Rapp salt-sensitive rats

Variable	*Dahl/Rapp on high-salt diet*	*Dahl/Rapp on high-salt diet + Levo 0.3*	*Dahl/Rapp on high-salt diet + Levo 3*	*Dahl/Rapp on low-salt diet*	*ANOVA P-value*
dU-Alb (mg days^{-1})	100.5 ± 14.8	106.8 ± 31.2	117.0 ± 12.3	52.4 ± 16.3	0.26
pl-BNP (pg ml^{-1})	27.3 ± 4.1	16.5 ± 1.3*	18.3 ± 1.9*	16.5 ± 1.2*	0.007
PRA (ng) Ang I (ml^{-1} h^{-1})	0.9 ± 0.2	3.9 ± 2.5	2.0 ± 0.7	1.2 ± 0.2	0.51
s-Aldosterone (pg ml^{-1})	116.0 ± 15.4	75.0 ± 18.3	118.8 ± 46.6	117.3 ± 10.4	0.64
dU-NA (nmol days^{-1})	1.92 ± 0.22	1.49 ± 0.26	1.08 ± 0.28*	1.73 ± 0.15	0.04

Abbreviations: dU-Alb, 24-h urinary albumin excretion; pl-BNP, plasma brain natriuretic peptide concentration; PRA, plasma renin activity; s-aldosterone, serum aldosterone concentration; dU-NA, 24-h urinary noradrenaline excretion.
Four experimental groups of animals were used: Dahl/Rapp rats on high-salt diet with and without levosimendan (Levo, 0.3 or 3 mg kg^{-1} via drinking fluid for 7 weeks) and Dahl/Rapp controls receiving low-salt diet. Variables measured were as follows: dU-Alb, pl-BNP, PRA, s-aldosterone, dU-NA. Data shown are means ± s.e.m., $n = 6$–12 in each group. *$P < 0.05$ compared to Dahl/Rapp rats on high-salt diet.

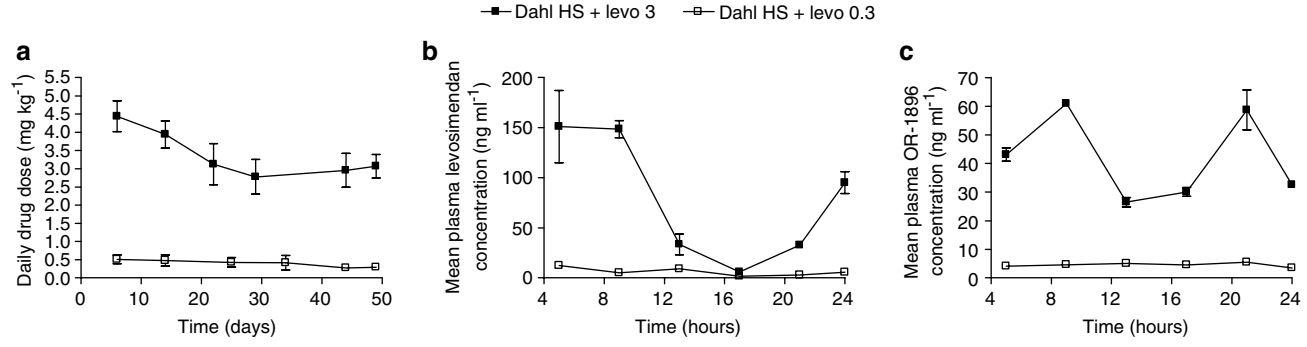

Figure 7 Line graphs showing average daily levosimendan doses (**a**) calculated from water consumption during the 7-week experimental period. Diurnal profiles of plasma levosimendan and OR-1896 are shown in (**b**) and (**c**), respectively. Solid squares denote Dahl/Rapp rats treated with high-dose levosimendan, solid triangles rats treated with low-dose levosimendan. Means ± s.e.m. are given. In (**a**), $n = 6$–12 in each group, in (**b** and **c**), $n = 4$ in each group. * denotes $P < 0.05$ compared to Dahl/Rapp rats on high-salt diet.

ATP-sensitive potassium channels (K_{ATP}) have been thought to be a mediator of cardioprotection in ischemic heart disease, and in particular, in ischemic–reperfusion injury (for reviews see Szewczyk and Marbán, 1999; Grover and Garlid, 2000; Ardehali and O'Rourke, 2005). It is believed that mitochondrial rather than sarcolemmal K_{ATP} channels mediate the physiological and molecular mechanisms of cardioprotection (Grover and Garlid, 2000). Although the signalling mechanisms that link mitochondrial K_{ATP} channel openers to cardioprotection are not completely characterized, Jaburek *et al.* (2006) provided compelling evidence that mitochondrial K_{ATP} channel and protein kinase C epsilon (PKCε) directly interact in the inner mitochondrial membrane, and that PKCε is required for the

opening of mitochondrial K_{ATP}. Levosimendan activates myocardial K_{ATP} channels at therapeutic plasma concentrations (Yokoshiki *et al*., 1997a, b; Kopustinskiene *et al*., 2001, 2004). Opening of K_{ATP} channels in ventricular cardiomyocytes has been shown to contribute to the inotropic action of levosimendan (Yokoshiki *et al*., 1997a). Levosimendan is a K_{ATP} channel opener of both plasma membranes and mitochondria. Hence, it is likely the vasodilatory and cardioprotective effects of oral levosimendan treatment found in the present study, are mediated partially via opening of the K_{ATP} channels.

Positive-inotropic agents such as phosphodiesterases (PDE) as well as β-adrenoceptor agonists are known to increase myocardial contractility through increased myocardial cyclic adenosine monophosphate (cAMP) levels and increased intracellular free calcium concentrations. Levosimendan displays structural similarities with a family of phosphodiesterase inhibitors and is also a very selective PDE III inhibitor (Szilagyi *et al*., 2004). In fact, high concentrations of levosimendan have been shown to inhibit PDE III and IV in human and guinea pig cardiomyocytes, and to stimulate L-type calcium current (Edes *et al*., 1995; Virag *et al*., 1996; Boknik *et al*., 1997; Ajiro *et al*., 2002; Szilagyi *et al*., 2004). Takahashi and Endoh (2005) have reported recently in experiments carried out in canine ventricular trabeculae that the muscarinic receptor agonist carbachol inhibited levosimendan-induced increase in calcium transients and abolished the positive-inotropic effect of the compound, suggesting that the increase in calcium transients induced by levosimendan is owing to its inhibition of PDE. On the contrary, Yokoshiki *et al*. (1997a) reported that levosimendan at concentrations varying from 0.2 to 10 μM did not affect calcium transients of rat cardiomyocytes. Such discrepancies might arise from the different species studied, which have different PDE isoenzymes. Taken together, it is possible that the positive inotropic action of levosimendan found in the present study, may have been partly mediated through phosphodiesterase inhibition.

Data from animal and cell culture experiments strongly support the notion that increased formation of ROS regulates apoptotic pathways in the heart (von Harsdorf *et al*., 1999; Giordano, 2005). Increased ROS formation plays an important role in the pathogenesis of target-organ damage in Dahl rats (Tojo *et al*., 2002). In good agreement with these findings we reported here that myocardial damage in Dahl rats is associated with increased perivascular inflammatory cell recruitment, expression of ROS-generating NADPH oxidase and induction of the gene expression of the redox-sensitive osteopontin. It is of particular interest, that opening of mitochondrial K_{ATP} channels inhibits ROS-induced apoptosis in myocytes (Akao *et al*., 2001). Kopustinskiene *et al*. (2004) have demonstrated that levosimendan activates potassium flux to the myocardial mitochondrial matrix at therapeutic plasma concentrations. Levosimendan also reduced the plasma levels of proinflammatory cytokines and soluble apoptosis mediators in patients with decompensated heart failure (Parissis *et al*., 2004). Furthermore, levosimendan, *in vitro,* protects cardiomyocytes from ROS-induced apoptosis via activation of K_{ATP} channels (Maytin and Colucci, 2005). It is of particular interest that PDE III inhibition with pharmacological agents, such as milrinone or cilostamide, promotes cardiomyocyte apoptosis (Ding *et al*., 2005). Taken together, our findings support the notion that the dose-dependent decrease in apoptotic cardiomyocytes by levosimendan found in the present study, was mediated by opening of the mitochondrial ATP-sensitive K^+ channels and inhibition of mitochondrial apoptotic pathway, and not by PDE III inhibition.

There is compelling evidence to indicate that altered intracellular calcium handling plays a key role in the development of heart failure (Bers, 2002; Hasenfuss and Pieske, 2002; Yano *et al*., 2005). It has been shown that cellular acidosis, typical of myocardial ischemia, consistently decreases calcium sensitivity of the cardiomyocytes owing to a decrease in the affinity of troponin C for Ca^{2+} (Takahashi and Endoh, 2005). Takahashi and Endoh (2005) reported recently that acidosis suppressed the inotropic effect of levosimendan *in vitro* owing to an attenuation of the increase in Ca^{2+} transients, whereas the increase by levosimendan in Ca^{2+} sensitivity remained even during acidosis. Involvement of impaired calcium handling in the pathogenesis was also suggested by Seki *et al*. (2003), who reported markedly decreased protein expression of SERCA2 and SERCA2/NCX ratio in hypertrophied heart from Dahl salt-sensitive rats. In very good agreement with these findings we found in the present study that levosimendan improved cardiac function, decreased SERCA2 protein expression and decreased myocardial SERCA2/NCX ratio.

Levosimendan treatment normalized the salt-induced increases in plasma BNP concentration and myocardial ANP mRNA expression, and slightly decreased urinary noradrenaline excretion suggesting amelioration of neurohumoral activation in Dahl/Rapp rats on high-salt diet. As BNP concentration correlates with increased end-diastolic pressure and left ventricular wall tension, our findings suggest that levosimendan was able to decrease myocardial tension and the degree of cardiac overload in Dahl/Rapp salt-sensitive rats, possibly leading to improved microcirculation in the heart.

Levosimendan did not exhibit a clear dose dependency in all parameters examined. The limitation of the present experimental procedure, which is suitable for clarifying the effects of oral levosimendan treatment on cardiac remodeling and cardiomyocyte apoptosis, is that it is unable to dissect the influence of calcium sensitization, K_{ATP} channel opening and PDE III inhibition, and thus to provide the exact molecular mechanism of action related to the beneficial cardiovascular effects of oral levosimendan treatment found in the present study. Therefore, further studies comparing the cardiovascular effects of K_{ATP} channel openers, inotropic agents not acting through calcium sensitization and PDE III inhibitors, with levosimendan are warranted.

In conclusion, using Dahl/Rapp salt-sensitive rats as an animal model of fulminant hypertension and hypertension-induced heart disease, we here demonstrated that oral treatment with levosimendan improved survival and prevented hypertension-induced cardiac remodeling, cardiomyocyte apoptosis and neurohumoral activation. Our findings suggest a therapeutic role for oral levosimendan in the prevention of hypertension-induced heart failure.

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Conflict of interest

The authors state no conflict of interest.

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