INTRODUCTION

Hypertension is the key risk factor for myocardial infarction, stroke and development of heart failure (1). Although the molecular mechanisms underlying the transition of cardiac hypertrophy to heart failure in hypertension remain partly unclear recent evidence suggests an important role for mechanical overload, neurohormonal factors and cytokine pathways leading to structural remodeling in the pathogenesis of hypertensive heart disease (2). In patients with decompensated heart failure classic inotropic agents such as beta-adrenergic agonists and phosphodiesterase III inhibitors provide usually short-term hemodynamic benefits, however, their long-term use has been correlated with poor survival rates (3). Calcium sensitizers comprise a new drug class that may offer hemodynamic and symptomatic improvements without increasing cAMP and intracellular calcium concentrations (4, 5). It has been suggested that calcium sensitizers could enhance contractility without a concurrent increase in the risk of cardiac events and thus may represent a significant improvement over classic positive inotropic agents. However, the clinical data so far are still controversial. We have shown recently using a widely used animal model for hypertensive heart disease, namely Dahl salt-sensitive rat on a high salt diet (6, 7) that chronic oral treatment with levosimendan is associated with improved outcome and amelioration of hypertensive myocardial remodeling (8). As compared with other calcium sensitizers levosimendan has a dual action; it is an inodilator acting both via calcium sensitization and opening of ATP-dependent potassium channels in the vasculature. OR-1896 (the (-) enantiomer of N-[4-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)phenyl] acetamide) is a biologically active metabolite of levosimendan. OR-1896 has been studied to a lesser extent than the parent compound; however, preliminary data suggest that OR-1896 exerts similar hemodynamic and pharmacological properties as levosimendan (9, 10). The aim of the present study was to investigate whether OR-1896 could prevent cardiovascular mortality and hypertension-induced myocardial remodelling in salt-sensitive forms of hypertension. The present study also underscores the importance of cellular senescence in the pathogenesis of salt-induced hypertensive heart disease.

EFFECTS OF CALCIUM SENSITIZER OR-1986 ON CARDIOVASCULAR MORTALITY AND MYOCARDIAL REMODELLING IN HYPERTENSIVE DAHL/RAPP RATS

Calcium-sensitizing agents have been shown to improve cardiac function in patients suffering from acute decompensated heart failure, however, their long-term effects on cardiac remodeling and cardiovascular mortality are still largely unknown. In the present study we tested the hypothesis whether OR-1896, an active and long-lasting metabolite of calcium sensitizer levosimendan, prevents cardiovascular mortality and hypertension-induced myocardial remodeling in salt-sensitive Dahl/Rapp rats. OR-1896 was given orally to Dahl/Rapp SS rats on high-salt diet (NaCl 7% w/w) for 7 weeks at two different doses (0.5 and 0.05 mg/kg). OR-1896 prevented salt-induced cardiovascular mortality (survival rate 75% in OR-1896 treated groups vs 38% in untreated controls, p<0.01), ameliorated cardiac hypertrophy and improved systolic functions of the heart without major influence on systemic blood pressure. OR-1896 also ameliorated salt-induced increase in cardiac ANP mRNA expression and plasma BNP level. Salt-induced cardiac remodelling was associated with 4-fold increase in cardiac p16INK4a mRNA expression, a marker of cellular senescence. OR-1896 dose-dependently ameliorated cardiomyocyte senescence. Our findings suggest a therapeutic role for OR-1896 in the prevention of cardiac remodelling in salt-sensitive forms of hypertension. The present study also underscores the importance of cellular senescence in the pathogenesis of salt-induced hypertensive heart disease.

Key words: calcium sensitizers, cardiac remodelling, hypertension, senescence
six 6-week-old male Dahl/Rapp salt sensitive rats (SS/JrHsd), purchased from Harlan (USA), were divided into four groups with following diet and drug regiments for 7 weeks: 1. Dahl/Rapp SS controls on high salt diet (NaCl 7 %) n=24, 2. Dahl/Rapp SS rats on high salt diet +OR-1896 0,5 mg/kg (high dose) n=12, 3. Dahl/Rapp SS rats on high salt diet +OR-1896 0,05 mg/kg (low dose) n=12 and 4. Dahl/Rapp SS rats on low salt diet n=6. To study the role of OR-1896 in beneficial effects of levosimendan found in previous studies by us and others (12) the dosage yielding corresponding plasma concentrations was chosen. In an additional pharmacokinetic experiment the diurnal blood drug concentrations of OR-1896 was examined after two weeks oral treatment with low-dose and high-dose OR-1896 (n=4 in both groups).

High salt diet was produced by adding NaCl (Riedel-de Haen AG, Seelze, Germany) to commercial low salt diet (Na 0.3 %, K 0.8 %, Mg 0.2 %; Harlan, Indianapolis, Indiana, USA). OR-1896 (Orion Pharma, Espoo, Finland) was given via drinking fluid using daily prepared solutions. 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). Urines were collected over a 24 h period in metabolic cages for albumin measurements. Urine volumes and water intakes were measured gravimetrically.

At the end of experiment rats were anesthetized with CO2/O2 (AGA, Riihimaki, Finland) and decapitated. Blood samples using EDTA as anticoagulant were collected for plasma BNP and aldosterone measurements. 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 12-MHz linear transducer under light isoflurane anesthesia on control rats at baseline, and all surviving at the end (7 weeks) of the experimental period as described previously (8).

**Heart and kidney morphology**

The arteries and ventricular fibrous tissue formation were evaluated in a blinded fashion and scores were from 0 to 5 according to morphological changes investigated as described in detail previously (8).

**P16\(^{INK4a}\) immunohistochemistry**

Immunoperoxidase staining for p16\(^{INK4a}\) was performed using 5 µm frozen sections. The sections were incubated for 30 minutes in a humid chamber at room temperature with 1:100 diluted primary monoclonal p16\(^{INK4a}\) antibody (Santa Cruz Biotechnologies, Santa Cruz, California, USA) and peroxidase-conjugated rabbit anti-mouse secondary antibody (DAKO A/S, Glostrup, Denmark). 3-amino-9-ethylcarbazole, was added to yield a red reaction product and finally the sections were slightly counterstained in Mayer’s haemaluna (Merek), blued in tap water.

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

Total RNA from the rat hearts were collected with Trizol (Invitrogen, Carlsbad, CA, USA), treated with DNAse 1 (Deoxyribonuclease 1, Sigma Chemicals Co., St Louis, MO, USA) and reverse transcribed to cDNA by incubation of 50 minutes in 45°C with presence of reverse transcription enzyme (Enhanced avian HS RT-PCR kit, Sigma Chemicals Co.). One µl of cDNA was subjected to a quantitative real time polymerase chain reaction by Lightcyler instrument (Roche diagnostics, Neuilly sur Seine, France) for detection of ANP, p16\(^{INK4a}\), p19\(^{ARF}\) and 18S mRNAs. 18S served as housekeeping gene. The samples were amplified by using FastStart DNA Master SYBR Green 1 (Roche diagnostics) in presence of 0.5 µM of following primers: ANP (13) forward CCGATAGATTCTGCCCTTGTGA, reverse CCGGAACGACCTTTGATCTTC ; p16\(^{INK4a}\) forward ACCAAAGCCGCGGAACA, reverse GACAGCTGCCATTTGACGT; p19\(^{ARF}\) forward, GAGGGCCGACGCACCAT, reverse CACCATAAGGAGGACAGGAGA; 18S (14) forward ACATCCAAGGAGAGCAGCAG, reverse TTTTCGTCACTACCTCCCGG. The PCR amplifications consisted of 10 minutes incubation in 95°C following 30 cycles of 15 seconds in 95°C, annealing for 5 seconds in 62°C and 10 seconds in 72 °C for ANP; 10 minutes incubation in 95°C following 45 cycles of 15 seconds in 95°C, annealing for 5 seconds in 60°C and 10 seconds in 72°C for p16\(^{INK4a}\); 10 minutes incubation in 95°C following 45 cycles of 15 seconds in 95°C, annealing for 5 seconds in 62°C and 10 seconds in 72°C for p19arf; 10 minutes incubation in 95°C following 28 cycles of 15 seconds in 95°C, annealing for 5 seconds in 63°C and 10 seconds in 72°C for 18S. The quantities of ANP, p16\(^{INK4a}\) and p19arf and 18S PCR products were quantified with an external standard curve amplified from purified PCR product.

**Biochemical determinations**

Urinary albumin was measured by ELISA using rat albumin as a standard (Celtrend, Luckenwalde, Germany). Plasma BNP (BNP-45, Peninsula Laboratories, San Carlos, CA, USA), PRA (Angiotensin I RIA kit, Diasorin, Inc., Salucci, Italy), and aldosterone (Coat-a-Count Aldosterone RIA kit, DPC, Los Angeles, USA) were determined by RIA according the instructions of the manufacturer. Urinary catecholamines were analyzed using the isocratic ion-pair reversed-phase high-pressure liquid chromatography (HPLC) method with electrochemical detection. Plasma concentrations of OR-1896 were measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

**Statistical analysis**

Data are presented as means ± SEM. Statistically significant differences in mean values were tested by analysis of variance (ANOVA) and Tukey’s post-hoc test. The differences were considered significant when p<0.05. Kaplan-Meier test was used for survival analysis.

**RESULTS**

**Survival rates**

High salt diet increased mortality of Dahl/Rapp rats; only 38% (9/24) of untreated Dahl/Rapp rats on high salt diet survived through 7 week study period, compared to 100% (6/6) of low salt Dahl/Rapp SS controls. (Kaplan Meier statistics p<0.01) OR-1896 increased survival rates in both dose groups; 75% (9/12) on high dose and 75% (9/12) on low dose (Kaplan–Meier statistics p<0.01 and p<0.01 compare to Dahl/Rapp rats on high salt diet, respectively) (Fig. 1).
**Systolic blood pressure, heart rate, morphology and hypertrophy**

High salt diet on Dahl/Rapp SS rats induces significant rise in blood pressure, already visible at 3.5 weeks, when compared to low salt controls (214 ± 5.8 mmHg vs. 148 ± 10, p<0.05) (Figs. 2A and 2B). Heart rate was also markedly elevated by high salt diet (442 ± 8 beats/min vs. 399 ± 4.2 mmHg, p<0.001) at the end of the experiment. High salt diet induces pronounced cardiac hypertrophy (5.0 ± 0.15 mg/g vs. 3.0 ± 0.07 mg/g, p<0.001) and cardiac damage, with intimal hyperplasia, fibrinoid necrosis of the arteries with medial thickening and adventitial scarring and occasional myocardial infarctions with inflammation (Figs. 2C, 2D, 3A and 3B). Dahl/Rapp rats on high salt diet also gained significantly less weight than low salt controls (278 ± 14.0 vs. 430 ± 5.6, p<0.001).

High dose OR-1896 produced a slight decrease in blood pressure level at midpoint of the experiment when compared to Dahl/Rapp SS on high salt diet (199 ± 2.8 vs. 181 ± 3.2, p<0.01). However, the blood pressure levels at endpoint of the experiment were identical in all high salt diet groups. Both OR-1896 treated groups showed less cardiac hypertrophy than Dahl/Rapp SS high salt controls when measured with heart weight to body weight ratio (Fig. 2C). Interestingly, OR-1896 was not able to decrease cardiac damage score (Fig. 2D).

**Echocardiography**

High salt diet induces moderate deterioration of cardiac functions and left ventricular hypertrophy in Dahl/Rapp rats when assessed by echocardiography. Ejection fraction and fractional shortening decreased in Dahl/Rapp rats on high salt diet. Higher dose of OR-1896 was able to improve contractility and normalize ejection fraction and fractional shortening even over the levels found in Dahl/Rapp rats low salt diet. Echocardiographic parameters are presented in Table 1.

**Cellular senescence: mRNA expressions of p16INK4a and p19arfl and p16INK4a immunohistochemistry**

High salt diet induced four fold change in the p16INK4a mRNA expression when compared to Dahl SS rats on low salt diet, while p19arfl expression was unaltered (Fig. 4). Both OR-1896 doses were able to reduce p16INK4a and increase p19arfl mRNA expressions in high salt Dahl SS rats. The p16INK4a positive staining in the cardiac samples was located in the nuclei of myocytes, arterial smooth muscle cells and to a lesser extent in the vascular endothelial cells. The perivascularly located leucocytes were also positive.

**Plasma BNP concentration and myocardial ANP mRNA expression**

Dahl/Rapp SS rats on high salt diet have markedly increased plasma BNP concentration and myocardial ANP mRNA expression.
expression than Dahl/Rapp rats on low salt diet. Both OR-1896 doses were able to normalize disturbances in expression of both natriuretic peptides, high dose even to the level found in Dahl/Rapp SS rats in low salt diet. (Fig. 5).

Biochemical determinations

Dahl/Rapp SS rats on high salt diet developed clear renal damage, measured by increased 24-hour albuminuria, but no

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**Table 1.** Echocardiographic parameters in Dahl/Rapp salt-sensitive rats on high salt diet, Dahl/Rapp rats treated with OR-1896 (0.05 mg/kg or 0.5 mg/kg via drinking fluid for 7 weeks) and Dahl/Rapp controls receiving low salt diet. Means ± SEM are given, n= 13-37 in each group. * p<0.05 compared to Dahl/Rapp rats on high salt diet.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Dahl/Rapp on high salt diet</th>
<th>Dahl/Rapp on high salt diet + OR-1896 high</th>
<th>Dahl/Rapp on high salt diet + OR-1896 low</th>
<th>Dahl/Rapp on low salt diet</th>
<th>ANOVA P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVD (d), mm</td>
<td>7.15 ± 0.16</td>
<td>7.50 ± 0.19</td>
<td>7.29 ± 0.16</td>
<td>8.31 ± 0.08 *</td>
<td>0.0353</td>
</tr>
<tr>
<td>LVD (s), mm</td>
<td>3.41 ± 0.18</td>
<td>2.63 ± 0.20 *</td>
<td>3.44 ± 0.26</td>
<td>3.42 ± 0.14</td>
<td>0.033</td>
</tr>
<tr>
<td>EDV, ml</td>
<td>0.95 ± 0.05</td>
<td>0.95 ± 0.06 *</td>
<td>0.88 ± 0.05</td>
<td>1.25 ± 0.03 *</td>
<td>0.0186</td>
</tr>
<tr>
<td>ESV, ml</td>
<td>0.01 ± 0.02</td>
<td>0.06 ± 0.01 *</td>
<td>0.12 ± 0.02</td>
<td>0.13 ± 0.02</td>
<td>0.059</td>
</tr>
<tr>
<td>SV, ml</td>
<td>0.73 ± 0.04</td>
<td>0.91 ± 0.05 *</td>
<td>0.75 ± 0.05</td>
<td>1.13 ± 0.04 *</td>
<td>0.0012</td>
</tr>
<tr>
<td>EF, %</td>
<td>85.6 ± 1.6</td>
<td>94.0 ± 1.0 *</td>
<td>86.2 ± 2.4</td>
<td>90.1 ± 1.7</td>
<td>0.0065</td>
</tr>
<tr>
<td>FS, %</td>
<td>52.9 ± 2.1</td>
<td>65.4 ± 2.2 *</td>
<td>53.1 ± 3.4</td>
<td>57.6 ± 2.1</td>
<td>0.0045</td>
</tr>
</tbody>
</table>

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**Fig. 3.** Representative photomicrographic graphs showing cardiac morphology and p16INK4a staining of Dahl/Rapp rats on high (Panels A and C) and low salt diet (Panels B and D). Original magnitude x10-40.

**Fig. 4.** Bar graphs showing hypertension induced activation of senescent signalling measured by cardiac p16INK4a and p19arf mRNA. OR-1896 treatment was able to suppress p16INK4a mRNA expression and increase expression of p19arf mRNA. Abbreviations see figure legend 2. Means±SEM are given, n=5-12 in each group. * denotes p<0.05 compared to Dahl/Rapp rats on high salt diet, # denotes p<0.05 when compared to Dahl/Rapp rats on low salt diet.
**Table 2.** Effects of OR-1896 on some biochemical and hormonal markers in Dahl salt-sensitive rats on high salt diet. OR-1896 was given orally at two doses for 7 weeks. Dahl SS rats on low salt diet served as controls. dU-Alb denotes 24-hour urinary albumin excretion; dU-V denotes daily urinary volume; dU-NA denotes daily urinary noradrenaline excretion; dU-Dopa denotes daily urinary dopamine excretion; PRA denotes plasma renin activity; s-Aldosterone denotes serum aldosterone concentration; KW/BW denotes kidney hypertrophy by kidney weight-body weight ratio. Means ± SEM are given, n=6-40 in each group. * p<0.05 compared to Dahl SS rats on high salt diet; # p<0.05 compared to Dahl SS rats on low salt diet.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Dahl/Rapp on high salt diet</th>
<th>Dahl/Rapp on high salt diet + OR-1896 High</th>
<th>Dahl/Rapp on high salt diet + OR-1896 Low</th>
<th>Dahl/Rapp on low salt diet</th>
<th>ANOVA P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRA, ng Ang 1 / ml/h</td>
<td>0.83 ± 0.2</td>
<td>1.73 ± 0.9</td>
<td>0.65 ± 0.08</td>
<td>1.1 ± 0.15</td>
<td>0.13</td>
</tr>
<tr>
<td>s-Aldosterone, pg/ml</td>
<td>116.0 ± 15.4</td>
<td>59.9 ± 9.9 * #</td>
<td>66.0 ± 9.3</td>
<td>117.3 ± 10.4</td>
<td>0.011</td>
</tr>
<tr>
<td>dU-NA, nmol/24 h</td>
<td>1.88 ± 0.22</td>
<td>1.40 ± 0.34</td>
<td>1.19 ± 0.36</td>
<td>2.20 ± 0.09</td>
<td>0.29</td>
</tr>
<tr>
<td>dU-Dopa, nmol/24 h</td>
<td>38.2 ± 3.1</td>
<td>61.6 ± 3.6 *</td>
<td>41.4 ± 6.1</td>
<td>53.9 ± 3.6</td>
<td>0.0034</td>
</tr>
<tr>
<td>dU-Ab 7 w, mg/d</td>
<td>113.3 ±44.2</td>
<td>116.0±28.0</td>
<td>106.7±55.5</td>
<td>52.4 ±36.4</td>
<td>0.092</td>
</tr>
<tr>
<td>dU-V 7 w, ml</td>
<td>56.5 ± 5.3</td>
<td>44.8 ± 8.0 #</td>
<td>47.8 ± 9.9 #</td>
<td>12.3 ± 2.7 *</td>
<td>0.00002</td>
</tr>
<tr>
<td>KW/BW, mg/g</td>
<td>6.0 ± 0.2</td>
<td>5.6 ± 0.3 #</td>
<td>5.5 ± 0.3 #</td>
<td>3.2 ± 0.1 *</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Observational studies have shown that blood pressure is strongly and directly related the relative risks of stroke and heart disease (15). Elevated blood pressure first leads to concentric left ventricular hypertrophy, which is considered as the heart’s adaptive response to a chronically increased workload. The precise molecular mechanisms mediating the transition of cardiac hypertrophy to heart failure are largely undefined. The important finding of the present study was the OR-1896, a novel inodilator acting mainly via calcium sensitization and opening of ATP-sensitive potassium channels, prevented cardiovascular mortality, improved cardiac functions, and ameliorated cardiac hypertrophy in hypertensive Dahl/Rapp rats on high salt diet. The beneficial effects of OR-1896 were largely independent of systemic blood pressure and associated with normalization of plasma BNP concentration and myocardial ANP mRNA expression, widely used markers of increased pressure/volume overload. We here also demonstrated that hypertensive heart disease in Dahl/Rapp rats was associated with increased cardiomyocyte senescence, which was dose-dependently prevented by oral OR-1896 treatment.

Dahl/Rapp SS rats are known to develop severe hypertension, moderate heart failure with preserved ejection failure (16) and end-organ damage resulting in overall 100 % mortality after 8 weeks on high salt diet. We showed recently that, levasimendan, a calcium sensitizer and ATP-potassium channel opener, improved survival in Dahl/Rapp rats on high salt diet without major changes in systemic blood pressure or heart rate (8). Levasimendan also improved systolic function, prevented cardiac remodeling, and dose-dependently decreased the number of apoptotic cardiomyocytes. These beneficial effects were associated with normalization of plasma BNP.
concentration, myocardial ANP mRNA expression, and SERCA2 expression of the heart (8). Levosimendan is completely metabolised in the body. In the rat, as well as in humans, only one active metabolite is formed. OR-1896, which has a longer elimination life than the parent compound (17) but exhibits similar hemodynamic effects (18). In details, OR-1896 is both a calcium sensitizer (9) and a vasodilator (19) as potent and effective as levosimendan. Since the free plasma concentrations of OR-1896 are comparable to those of the parent compound it is likely that this metabolite is involved in the cardiovascular effects that develop following levosimendan administration in therapy (20).

The primary goal of this study was to evaluate whether the active long-lasting metabolite could protect against hypertension-induced cardiovascular mortality and target-organ damage as the parent compound. The OR-1896 dosages used in the present study for Dahl/Rapp rats (0.05 and 0.5 mg/kg) produced plasma OR-1896 concentrations similar to those found in our previous study with oral levosimendan treatment conducted in Dahl/Rapp rats. Such plasma OR-1896 concentrations can also been found in patients with acute decompensated heart failure treated with intravenous levosimendan. The diurnal drug plasma concentration analysis did not reveal any major fluctuation of drug concentration. The important finding of the present study was that OR-1896 prevented effectively cardiovascular mortality in Dahl/Rapp rats, ameliorated cardiac hypertrophy, improved cardiac function and attenuated the neurohumoral activation without major changes in systemic blood pressure. We noticed a modest transient decrease in systolic blood pressure in the beginning of the study which might explain, to a minor extent, the beneficial effect of OR-1896 on left ventricular mass. However, it should be underlined, that in the present study the late-treatment with OR-1896 also prevented cardiovascular mortality in Dahl/Rapp rats with established hypertension and target-organ damage suggesting involvement of blood-pressure independent mechanisms (data not shown). OR-1896 did not provide any significant renoprotection when assessed by albuminuria and tissue morphology indicating that the beneficial effect by OR-1896 on cardiovascular mortality was not due to prevention of renal failure. It is well known that stroke and cerebral haemorrhages are also possible causes of death in Dahl/Rapp rats (21). As our study did not focus on cerebral morphology, we cannot fully exclude the possibility that prevention of cardiovascular mortality by OR-1896 was partially due to prevention of hypertension-induced cerebral complications.

In the present study a marked difference in mortality was noted between levosimendan-treated and untreated Dahl/Rapp rats on high salt diet. Since untreated Dahl/Rapp rats showed only a modest decrease in ejection fraction indicating preserved systolic function, other causes of death than heart failure should also be considered. When kept on high salt diet Dahl/Rapp rats develop very early left ventricular hypertrophy (LVH), the primary cardiac manifestation of hypertension. Clinical studies have revealed that LVH is a powerful risk factor for myocardial infarction, congestive heart failure, and cardiac arrhythmias, in particular sudden cardiac death (22, 23). Hypertensive patients with LVH have significantly greater prevalence of premature ventricular contractions and complex ventricular arrhythmias compared to patients without LVH or normotensive patients (22, 23). The increase in cardiac mass lowers coronary reserve and enhances cardiac oxygen requirements giving rise to ventricular ectopy. It is therefore likely that increased mortality in hypertensive Dahl Rapp rats found in the present study could be explained, at least in part, by sudden cardiac death due to ventricular arrhythmias. A recent report by Kamei et al (24) demonstrating that Dahl salt-sensitive rats exert increased susceptibility to ventricular tachycardia after external stimuli, support our notion. Unfortunately data on the effects of OR-1896 on cardiac arrhythmias are non-existent, however, the parent compound levosimendan, to which OR-1896 shows striking pharmacological similarities (10, 12, 19, 25), has been shown to prevent post-ischemic ventricular tachyarrhythmia in guinea pigs and rabbits (26, 27). It is therefore tempting to speculate that the beneficial effect of OR-1896 on mortality in hypertensive Dahl/Rapp rats on high salt diet could have been linked to anti-arrhythmic properties of OR-1896. Further studies are therefore warranted to investigate the effects of OR-1896 on ventricular arrhythmias in hypertensive Dahl/Rapp rats with pronounced left ventricular hypertrophy.

Two products of INK4a/ARF locus, cyclin cycle inhibitor p16INK4a and p53 activator p19ARF, creates unique system for mediating cellular senescence and suppressing malignancy. Increased p16INK4a expression has been found in several mammalian organisms and pathological conditions, including native kidney diseases and aging human and mouse hearts. Human hearts with premature cardiac aging and heart failure show accumulation of p16INK4a positive primitive cells and myocytes combined with shortened telomeres reflecting the imbalance between cellular growth and death (28). Although both p16INK4a and p19ARF are associated with cellular senescence, their actions on aging seem to be different. Using BubR1-insufficient mice as model of cellular aging Baker et al. showed very recently that p16INK4a promotes cellular cencesence whereas p19ARF in fact exerts anti-promoting effects on cellular aging (29). It is of great interest that hypertensive heart disease is also associated with increased cellular senescence. Westhoff et al. showed very recently the first evidence that hypertension induces cardiomyocyte senescence via mechanisms linked to increased renin-angiotensin system activity (11). In good accordance with Westhoff et al. we showed here using a low-renin animal model of hypertension that p16INK4a expression in the heart is increased by four-fold compared to low-salt controls. Although the myocardial expression of p19ARF was also increased by 40 %; however this difference did not reach statistical significance. We were able to demonstrate for the first time that OR-1896 suppressed cardiomyocyte senescence as indicated by a drug-induced decrease in expression of pro-aging p16INK4a and increased expression of anti-aging p19ARF in the heart. Using immohistochemistry we could confirm the nuclear localization of p16INK4a and p19ARF mainly in the cardiomyocytes. Our findings also suggest that the influence of OR-1896 on cellular senescence was primarily due to blood-pressure independent mechanisms. It has been shown very recently that silostazol, a selective PDE III inhibitor, prevented oxidative stress-induced premature aging of endothelial cells (30). It is therefore tempting to speculate that the beneficial effects of OR-1896 on cardiomyocyte cellular senescence could be related to anti-oxidative properties of OR-1896 or to PDE III inhibition, as OR-1896 like the parent compound levosimendan, inhibit PDE III selectively at low nanomolar range. However, further studies are needed to evaluate the molecular mechanisms of cellular senescence in the heart and in particular the possible pathways linked to OR-1896.

Acknowledgements: This study was supported by grants from Academy of Finland, University of Helsinki, Sigrid Juselius Foundation, Paiviikki and Sakari Sohlberg’s Foundation, Orion-Farmos Research Foundation and the Finnish Foundation of Cardiovascular Research. We are grateful for Ms. Anneli von Behr and Ms. Sari Laakkonen for their excellent technical assistance.

Conflict of interests: None declared.
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Received: September 26, 2008
Accepted: July 15, 2009

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