BJP BRITISH JOURNAL OF PHARMACOLOGY

BPS BRITISH PHARMACOLOGICAL SOCIETY

British Journal of Pharmacology (2010), 160, 142–152

0007-1188/10
www.brjpharmacol.org

RESEARCH PAPER

Effects of the calcium sensitizer OR-1896, a metabolite of levosimendan, on post-infarct heart failure and cardiac remodelling in diabetic Goto–Kakizaki rats

Marjut Louhelainen[1], Saara Merasto[1], Piet Finckenberg[1], Erik Vahtola[1], Petri Kaheinen[1], Jouko Levijoki[2] and Eero Mervaala[1]

[1]*Institute of Biomedicine, Pharmacology, University of Helsinki, Helsinki, Finland, and* [2]*Orion Pharma Ltd, Espoo, Finland*

Background and purpose: Levosimendan is a novel, short half-life calcium sensitizer used as pharmacological inotropic support in acute decompensated heart failure. After oral administration, levosimendan is metabolized to OR-1855, which, in rats, is further metabolized into OR-1896. OR-1896 is a long-lasting metabolite of levosimendan sharing the pharmacological properties of the parent compound.
Experimental approach: Effects of oral OR-1896 treatment on post-infarct heart failure and cardiac remodelling were assessed in diabetic Goto–Kakizaki (GK) rats, an animal model of type II diabetes. Myocardial infarction (MI) was produced to GK rats by coronary ligation. Twenty-four hours after MI or sham operation, the rats were randomized into four groups: (i) MI; (ii) MI + OR-1896 treatment; (iii) sham; and (iv) sham + OR-1896. Cardiac function and markers of cardiac remodelling were assessed 1, 4 and 12 weeks after MI.
Key results: OR-1896 increased ejection fraction and fractional shortening in GK rats with MI. OR-1896 ameliorated post-infarct cardiac hypertrophy, and prevented the MI-induced increase in cardiac mRNA for atrial natriuretic peptide, monocyte chemoattractant protein-1 and connective tissue growth factor, markers of pressure/volume overload, inflammation and fibrosis respectively. OR-1896 also suppressed mRNA for senescence-associated $p16^{INK4A}$ and $p19^{ARF}$. The beneficial effects of OR-1896 were more prominent at week 12 than at week 4. OR-1896 did not influence systolic blood pressure, blood glucose level, myocardial infarct size or cardiovascular mortality.
Conclusions and implications: Oral treatment with calcium sensitizer OR-1896 protects against post-infarct heart failure and cardiac remodelling in experimental model of type II diabetes.
British Journal of Pharmacology (2010) **160,** 142–152; doi:10.1111/j.1476-5381.2010.00680.x; published online 23 March 2010

Keywords: calcium sensitizer; heart failure; cardiac remodelling; senescence

Abbreviations: ANP, atrial natriuretic peptide; CTGF, connective tissue growth factor; GK, Goto–Kakizaki; IL-6, interleukin-6; MCP-1, monocyte chemoattractant protein-1; MI, myocardial infarction; NCX, sodium/calcium exchanger; NR4a3, nuclear receptor 4a3; NRF-1, nuclear respiratory factor-1; PCG-1α, PPARγ co-activator-1; SERCA2, sarco-/endoplasmic reticulum Ca^{2+}-ATPase 2a; TFAM, transcription factor A, mitochondria; VEGF, vascular endothelial growth factor

Introduction

Calcium sensitizers comprise a novel class of positive inotropic agents that may possess potential advantages for the treatment of acute decompensated heart failure, compared with conventional cardiotonic drugs (Endoh, 2008). The calcium sensitizer levosimendan has been shown to reduce peripheral vascular resistance, and enhance the contractility of the failing heart, without significantly increasing myocardial oxygen uptake (Innes and Wagstaff, 2003). The cardiovascular effects of levosimendan are mediated mainly by two mechanisms of action: (i) calcium sensitization via binding to the Ca^{2+}-saturated troponin C (cTnC) in cardiomyocytes; and (ii) opening of the ATP-sensitive potassium channels on the sarcolemma and mitochondria (Haikala *et al.*, 1995; Bowman *et al.*, 1999; Sorsa *et al.*, 2001;

Correspondence: Eero Mervaala, Institute of Biomedicine, Pharmacology, PO Box 63, FI-00014 University of Helsinki, Helsinki, Finland. E-mail: eero.mervaala@helsinki.fi
Received 21 September 2009; revised 8 December 2009; accepted 3 January 2010

Kopustinskiene *et al.*, 2004;Ozdem *et al.*, 2006; du Toit *et al.*, 2008).

Levosimendan has a short elimination half-life of approximately 1 h in humans (Sandell *et al.*, 1995) and 0.7 h in rats (Orion Pharma, unpubl. info). The circulating active metabolites OR-1855 and OR-1896 are formed slowly. In rats, OR-1855 is very extensively and rapidly transformed into OR-1896. OR-1896 has a longer elimination half-life than the parent compound levosimendan (elimination half-life of OR-1896 is 75–78 h in humans, 6.5 h in rats). It has been shown previously that OR-1896 exhibits haemodynamic effects similar to those of levosimendan. OR-1896 has been shown to act as a calcium sensitizer to elicit vasodilatation, and to inhibit PDE III *in vitro* (Takahashi *et al.*, 2000; Takahashi and Endoh, 2002; Szilagyi *et al.*, 2004; Erdei *et al.*, 2006; Banfor *et al.*, 2008; Segreti *et al.*, 2008). Hence, it is evident that the long-term cardiovascular effects after short-term levosimendan administration are attributed also to its long-lived metabolite OR-1896.

The present study aimed at exploring the effects of OR-1896 in the treatment of post-infarct heart failure and cardiac remodelling. As diabetes is a well-known risk factor for fatal myocardial infarction (MI) and development of heart failure, we investigated the cardiovascular effects of long-term oral OR-1896 therapy in an experimental model of MI and type II diabetes, the spontaneously diabetic Goto–Kakizaki (GK) rat (Galli *et al.*, 1999) with MI produced by ligation of the left descending coronary artery.

Methods

Experimental animals and experimental MI

All animal care and experimental protocols conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85–23, revised 1996), and were approved by the Animal Experimentation Committee of the University of Helsinki, Helsinki, Finland, and the Provincial State Office of Southern Finland (approval number STU 1187 A). Experimental MI was produced by ligating the left anterior descending (LAD) coronary artery in 8-week-old spontaneously diabetic GK rats (M & B, Ejby, Denmark) as previously described (Palojoki *et al.*, 2001a) under ketamine (65 mg·kg^{-1}, i.p.) and medetomidine (0.5 mg·kg^{-1}, i.p.) anaesthesia. Long-acting insulin (1 IU/rat) was given 2 h before anaesthesia to prevent hyperglycaemia. Buprenorphine (0.02 mg·kg^{-1} s.c.) was given twice a day for two consecutive days as the post-operative analgaesia. Twenty-four hours after surgery, MI and sham-operated GK rats were randomized into four groups: (i) GK rat with MI (n = 21); (ii) GK rat with OR-1896 (n = 16); (iii) GK rat with sham operation (n = 16); and (iv) GK rat with sham operation and OR-1896 (n = 12). Half of the rats in each study group were killed 4 weeks post-MI, and the remaining rats 12 weeks post-MI. As vasodilation and thus decreased peripheral resistance may influence cardiac functions measured at systole, we also investigated in a separate experiment the haemodynamic effects of angiotensin receptor antagonist valsartan (30 mg·kg^{-1} per os for 4 weeks) GK rats with MI (n = 6 GK rats with MI, n = 6 GK rats with MI treated with valsartan). OR-1896 was given in drinking water (0.5 mg·L^{-1}) to produce an approximate daily dose of 40 μg·kg^{-1}. The OR-1896 dosage was chosen based on the plasma OR-1896 levels detected after oral levosimendan administration in our previous experiments (Levijoki *et al.*, 2001; Louhelainen *et al.*, 2007; 2009).

Blood pressure measurement and sample preparation

Systolic blood pressure was measured using a tail cuff blood pressure analyser (Apollo-2AB Blood Pressure Analyzer, model 179-2AB, IITC Life Science, Woodland Hills, CA, USA) at weeks 1, 4 and 12. Rats were anaesthetized with CO_2/O_2 (AGA, Riihimäki, Finland), and decapitated. Blood samples were collected for biochemical measurements using EDTA as an anticoagulant. The hearts were excised, washed with ice-cold saline, blotted dry, weighed and snap-frozen in liquid nitrogen or isopentane (–35°C). All samples were stored at –80°C until assayed.

Infarct size, cardiac hypertrophy and cardiomyocyte cross-sectional area

For histological analysis, the hearts were fixed in 10% buffered formalin solution. The infarct sizes were determined planimetrically from paraffin-embedded 5 μm thick Masson's trichrome-stained histological sections as previously described (Pfeffer *et al.*, 1979; Palojoki *et al.*, 2001b). Conventional light microscopy at ×400 magnification was used to determine cardiomyocyte cross-sectional area as previously described (Vahtola *et al.*, 2008).

Echocardiography

Transthoracic echocardiography (Toshiba Ultrasound, Tokyo, Japan) was performed 1, 4 and 12 weeks after MI on all rats under isoflurane anaesthesia (AGA), without knowledge of the treatments, by the same technician as previously described (Louhelainen *et al.*, 2007; Vahtola *et al.*, 2008).

C-kit and p16^{INK4A} immunohistochemistry

C-kit-positive stem cells were identified from sections from paraffin-embedded heart samples, immunostained by standard ABC technique using c-kit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) with antigen retrieval.

Immunoperoxidase staining for the senescence marker p16^{INK4A} was performed using 5 μm frozen sections. Primary monoclonal p16^{INK4A} antibody (Santa Cruz Biotechnology) and peroxidase-conjugated rabbit anti-mouse secondary antibody (DAKO A/S, Glostrup, Denmark) were used. 3-Amino-9-ethylcarbazole was added to yield a red reaction product, and finally the sections were slightly counterstained in Mayer's haemalum (Merck, Darmstadt, Germany), blued in tap water.

Quantitative real-time RT-PCR

Total RNA from rat hearts was collected with Trizol (Gibco, Invitrogen, Carlsbad, CA, USA), treated with DNAse 1

(deoxyribonuclease 1, Sigma Chemicals Co., St Louis, MO, USA) and reverse transcribed to cDNA by reverse transcription enzyme (Im-Prom-II reverse trascription system, Promega, Madison, WI, USA). One microlitre of cDNA was subjected to quantitative real-time PCR using the LightCycler instrument (Roche Diagnostics, Neuilly-sur-Seine, France) for detection of atrial natriuretic peptide (ANP) (Heyen *et al*., 2002), connective tissue growth factor (CTGF), vascular endothelial growth factor (VEGF), nuclear receptor NR4a3, monocyte chemoattractant protein-1 (MCP-1), $p16^{INK4A}$ (Bastide *et al*., 2008), $p19^{ARF}$ (Bastide *et al*., 2008), transcription factor E2F-5, nuclear respiratory factor 1 (NRF-1), transcription factor A, mitochondria (TFAM), PPARγ coactivator-1 (PGC-1), sarco-/endoplasmic reticulum Ca^{2+}-ATPase 2a (SERCA2) (Louhelainen *et al*., 2007), sodium/calcium exchanger (NCX) (Louhelainen *et al*., 2007) and ribosomal 18S (Wellner *et al*., 2005) mRNA. The following primers were used: CTGF forward GGCAGGGCCAACCACTGTGC, reverse CAGTGCACTTGCCTGGATGG; VEGF forward GCGAGGCAGTTGAGT, reverse GGCGAATCCAGTTCCACG; NR4a3 forward AGCatTGGGTGACTCGat, reverse AACACGGAATGTTGAACCGTA; MCP-1 forward GCAGGTCTCTGTCACGCTTCT, reverse GGCTGAGACAGCACGTGGAT; E2F-5 forward GGACCAATCCATGTGC, reverse AGGAGACAGCCGTAAG; NRF-1 forward GCACCGTGTCGCTCAT, reverse GCTTGCGTCGTCTGGAT; TFAM forward AGACCTCGGTCAGCATATAACA, reverse GCGACGGATGAGATCACTT; PGC-1 forward GGTCCCCAGGCAGTAG, reverse CTCCatCatCCCGCAG.

The samples were amplified using FastStart DNA Master SYBR Green 1 (Roche Diagnostics) according to the protocol of the manufacturer.

Biochemical analyses

Blood glucose was determined with a hand-held test meter (Contour, Bayer Diabetes Care, Basel, Switzerland), plasma renin activity (PRA; angiotensin I RIA kit, Diasorin, Saluggia, Italy), serum aldosterone (Coat-a-Count Aldosterone RIA kit, DPC Biermann, Bad Nauheim, Germany) and serum insulin (rat insulin RIA kit, Linco, St Charles, MO, USA) were determined by radioimmunoassay according to the manufacturers' instructions. Plasma samples were analysed for OR-1896 by liquid chromatography–tandem mass spectrometry (Kivikko *et al*., 2003).

Statistical analyses

Data are presented as means ± SEM. Statistically significant differences in mean values were tested by ANOVA and Bonferroni's *post hoc* test for comparisons of multiple groups. The differences were considered significant when $P < 0.05$.

Materials

Valsartan and medetomidine were from Orion Pharma, Espoo, Finland; buprenorphine was from Schering-Plough, Brussels, Belgium; long-acting insulin was from Novo Nordisk A/S, Bagsvaerd, Denmark; and ketamine was from Pfizer Ltd, Helsinki, Finland.

Results

MI, blood pressure and echocardiography

Mortality for MI during the first 24 h prior to randomization was 60%. During follow-up period, only seven rats died (three sham, three MI and 1 MI + OR-1896), mostly in the early weeks after operation. There were no differences in infarct size between the MI groups measured by planimetry; the average infarct size was 32%. Compared to sham-operated rats, MI resulted in 15 and 25 mm Hg lower blood pressure 1 and 4 weeks after the operation, respectively (Figure 1A). Oral treatment of OR-1896 had no effect on blood pressure in GK rats with MI, whereas in sham-operated GK rats OR-1896 decreased blood pressure by approximately 30 mm Hg at weeks 4 and 12 after a modest and transient increase in blood pressure found 1 week after the operation. Heart rate in GK rats with MI was lower than in sham-operated GK controls. OR-1896 increased heart rate in GK rats with MI, whereas heart rate in sham-operated GK rats was not influenced by OR-1896 treatment (Figure 1B).

Baseline echocardiography values before operations were taken from six GK rats, and data from these measurements are given in Table 1. In GK rats after MI, serial echocardiography measurements taken at weeks 1, 4 and 12 revealed severe post-infarct systolic heart failure measured by decreased ejection fraction and fractional shortening (Figure 1C,D; Table 2). Oral OR-1896 treatment improved ejection fraction and fractional shortening, more obviously at the end of the follow-up period. In sham-operated GK rats, OR-1896 increased ejection fraction and fractional shortening at week 1, but not at week 4 or 12.

Cardiomyocyte hypertrophy

MI induced cardiac hypertrophy measured by heart weight/body weight ratio (Figure 2A and C) and cardiomyocyte cross-sectional area (Figure 2B and D). OR-1896 treatment decreased MI-induced cardiac hypertrophy. OR-1896 also prevented MI-induced increase in cardiomyocyte cross-sectional area; however, this effect was statistically significant only at week 12 (Figure 2D). In sham-operated GK rats, OR-1896 did not influence cardiac hypertrophy, but it slightly decreased cardiomyocyte cross-sectional area at the end of the follow-up period.

Expression of neurohumoral, inflammatory and fibrotic markers

Expression of mRNA for ANP was increased by threefold after MI (Figure 3A). Oral OR-1896 prevented MI-induced increase in cardiac ANP mRNA expression. OR-1896 reduced ANP mRNA expression in sham-operated GK rats. MCP-1 mRNA expression was measured as a marker of oxidative stress-induced inflammation. MCP-1 mRNA expression was increased by 3.5-fold in GK rats with MI (Figure 3B). OR-1896 treatment prevented MI-induced increase in cardiac MCP-1 mRNA expression.

MI induced permanent rise in the mRNA expression of CTGF, a potent profibrogenic growth factor acting downstream of transforming growth factor-β1 (Figure 3C). OR-1896

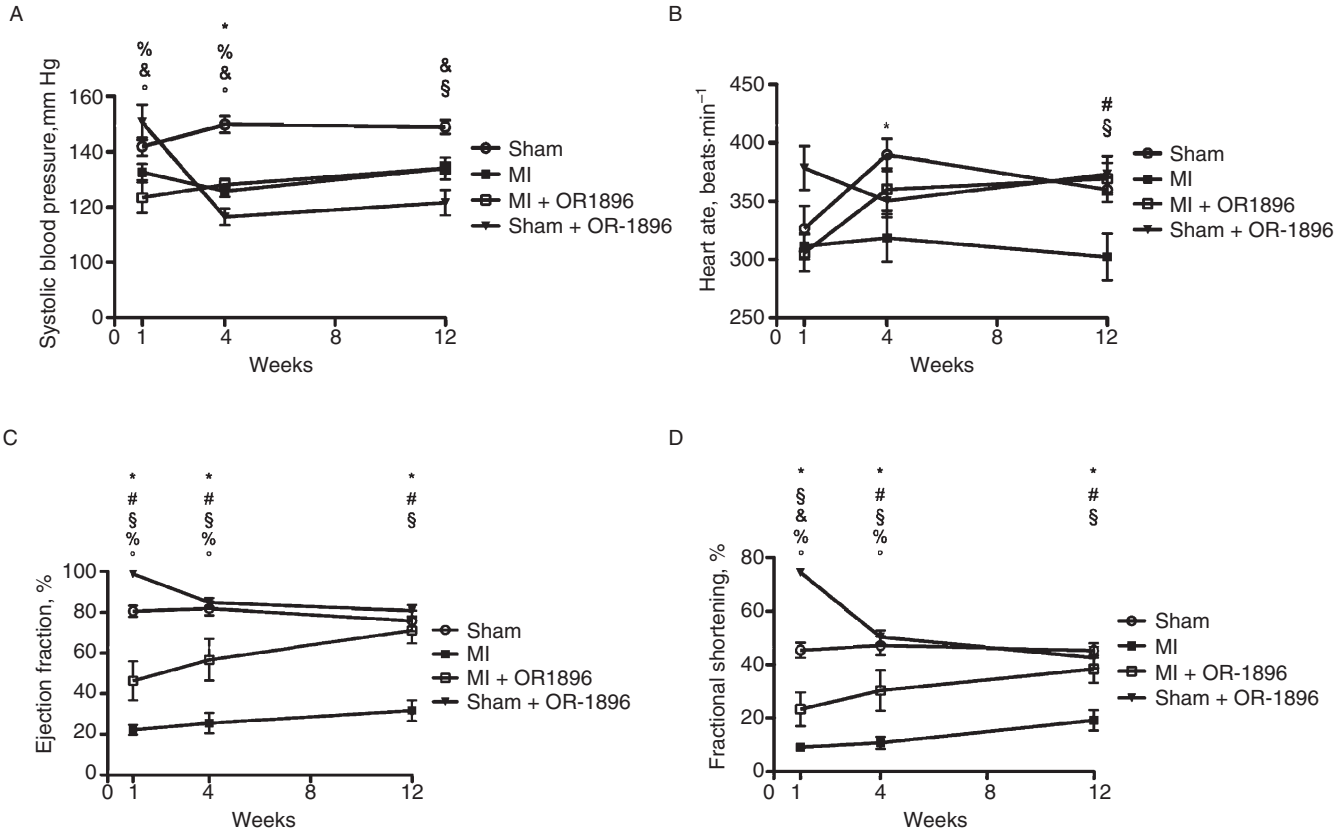

Figure 1 Effects of OR-1896 treatment on blood pressure (A), heart rate (B), ejection fraction (C) and fractional shortening (D) in spontaneously diabetic GK rats 1, 4 and 12 weeks after MI or sham operation. MI: GK rats with acute MI, MI + OR-1896; GK rats with MI and oral OR-1896 treatment; sham: GK rats with sham operation, sham + OR-1896; GK rats with sham operation and oral OR-1896 treatment. Means ± SEM are given, $n = 6$–12 in each group. *$P < 0.05$ when compared MI versus sham, #$P < 0.05$ MI versus MI + OR-1896, §$P < 0.05$ MI versus sham + OR-1896, %$P < 0.05$ sham versus MI + OR-1896, &$P < 0.05$ sham versus sham + OR-1896, °$P < 0.05$ MI + OR-1896 versus sham + OR-1896. All comparisons were made to age-matched animals.

Table 1 Baseline cardiac functions measured by echocardiography in spontaneously diabetic GK rats before MI

Variable	*GK rat*
IVS (d), mm	1.48 ± 0.07
LVD (d), mm	7.12 ± 0.2
LVW (d), mm	1.87 ± 0.09
EDV, mL	0.83 ± 0.06
ESV, mL	0.17 ± 0.02
EF, %	79.8 ± 1.86
FS, %	44.0 ± 2.02
CO, mL·min^{-1}	212.5 ± 21.4
HR, beats·min^{-1}	331 ± 14
BP, mm Hg	142 ± 2.9

Data in the table are means ± SEM, $n = 6$ in each group.
d, diastolic; IVS, interventricular septum; LVD, left ventricular diameter; LVW, left ventricular wall; EDV, end diastolic volume; ESV, end systolic volume; EF, ejection fraction; FS, fractional shortening; CO, cardiac output; HR, heart rate.

prevented MI-induced increase in cardiac CTGF mRNA expression. In sham-operated GK rats, OR-1896 tended to decrease cardiac MCP-1 and CTGF mRNA expressions; however, these differences did not reach statistical difference.

Neither MI nor OR-1896 influenced cardiac SERCA2 or NCX mRNA expression in GK rats (Figure 4).

VEGF, NR4a3 and E2F-5 mRNA expression

Cardiac VEGF, NR4a3 and E2F-5 mRNA expressions were increased only at the late stage of post-infarct cardiac remodelling (Figure 5A, C and D). OR-1896 prevented MI-induced increases in cardiac VEGF, NR4a3 and E2F-5 mRNA expressions, but did not influence their expression in sham-operated GK rats.

Cardiomyocyte senescence

MI induced a permanent increase in myocardial $p19^{ARF}$ mRNA expression, while $p16^{INK4A}$ mRNA expression was significantly up-regulated only at late stage of post-infarct remodelling (Figure 6A and B). Oral OR-1896 treatment normalized myocardial $p19^{ARF}$ mRNA expression and prevented up-regulation of myocardial $p16^{INK4A}$; $p16^{INK4A}$ was also detected by immunohistochemistry in cardiomyocytes and fibroblasts.

Mitochondrial biogenesis markers

Cardiac mRNA expressions of PGC, NRF-1 and TFAM were measured as markers of mitochondrial biogenesis. MI significantly induced NFR-1 mRNA expression 12 weeks post-MI, while similar trend towards increased expressions was seen also with PGC-1 and TFAM (Figure 7A–C). OR-1896 prevented MI-induced changes in the mitochondrial biogenesis markers.

Table 2 Echocardiographic parameters in GK rats measured 1, 4 and 12 weeks after MI

Variable	*GK MI 1 week*	*GK MI + OR-1896 1 week*	*GK sham 1 week*	*GK sham + OR-1896 1 week*	*anova P value*
CO, mL	122.6 ± 14.0	255.1 ± 23.8&	185.8 ± 17.7*	252.6 ± 22.4*#	<0.0001
ESV, mL	1.4 ± 0.02	0.67 ± 0.2*&	0.14 ± 0.02*	0.01 ± 0.005*	<0.0001
LVD, (d) mm	9.5 ± 0.2	8.4 ± 0.5*&	6.7 ± 0.1*	6.1 ± 0.2*#	<0.0001
Variable	*GK MI 4 weeks*	*GK MI + OR-1896 4 weeks*	*GK sham 4 weeks*	*GK sham + OR-1896 4 weeks*	*anova P value*
CO, mL	158.7 ± 34.0	199.2 ± 41.2	221.1 ± 16.7	232.1 ± 22.2	0.081
ESV, mL	1.8 ± 0.2	0.67 ± 0.24*	0.15 ± 0.05*	0.16 ± 0.02*	<0.0001
LVD, (d) mm	10.3 ± 0.3	8.5 ± 0.5*#	6.7 ± 0.3*	6.9 ± 0.18*#	<0.0001
Variable	*GK MI 12 weeks*	*GK MI + OR-1896 12 weeks*	*GK sham 12 weeks*	*GK sham + OR-1896 12 weeks*	*anova P value*
CO, mL	228.4 ± 21.9	355.4 ± 30*	321.2 ± 23.71*	251.5 ± 45.3	0.0048
ESV, mL	1.44 ± 0.3	0.70 ± 0.25	0.22 ± 0.03*	0.12 ± 0.02&	<0.0001
LVD, (d) mm	9.3 ± 0.7	10.0 ± 0.6	7.9 ± 0.2*	7.0 ± 0.31*#	0.0006

OR-1896 was given orally at a daily dose of 0.5 mg·kg^{-1}.
Data in the table are means ± SEM; n = 6–17 in each group.
*P < 0.05 compared to GK MI.
&P < 0.05 compared to GK sham.
#P < 0.05 compared to GK MI + OR-1896.
s, systolic; d, diastolic; EF, ejection fraction; FS, fractional shortening; CO, cardiac output; ESV, end systolic volume; LVD, left ventricular diameter.

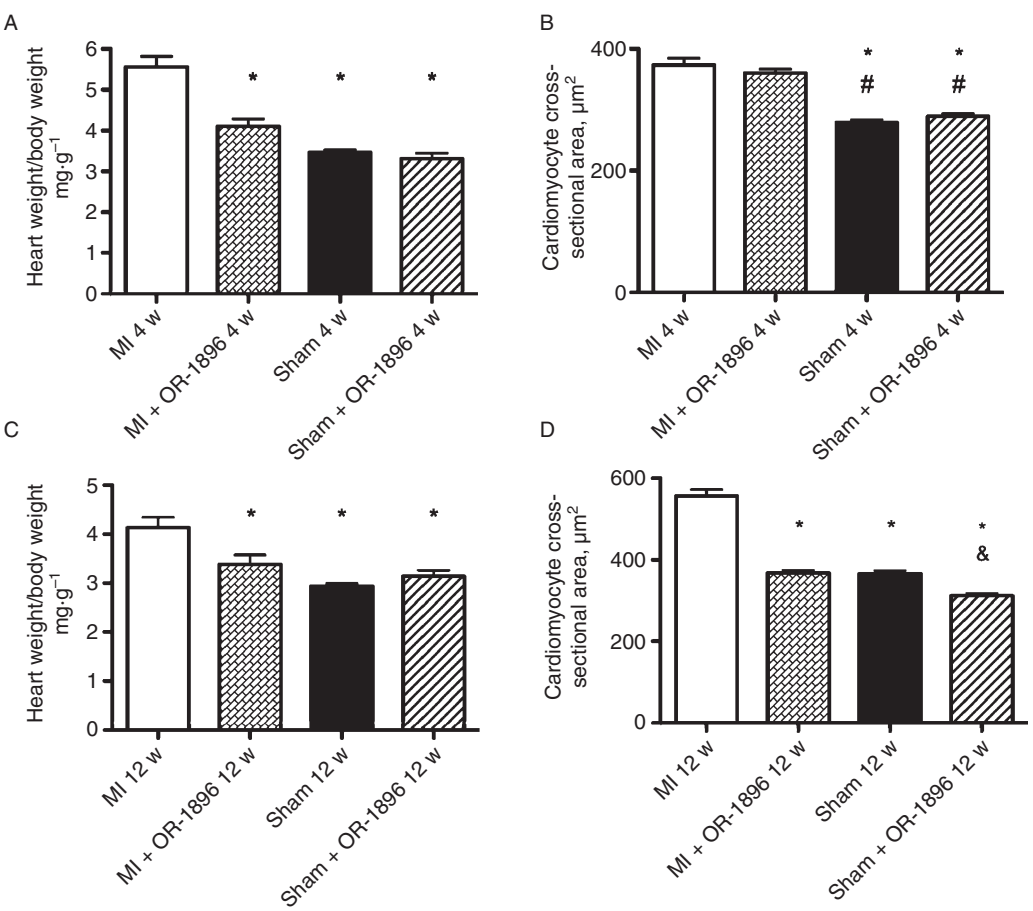

Figure 2 Effects of oral OR-1896 treatment on cardiac hypertrophy measured as heart weight-to-body weight ratio (A, C) and cardiomyocyte cross-sectional area (B, D) in spontaneously diabetic GK rats 4 and 12 weeks after MI or sham operation. Abbreviations for experimental groups as in Figure 1. Means ± SEM are given, n = 6–12 in each group. *P < 0.05 when compared to age-matched GK MI, #P < 0.05 to age-matched MI + OR-1896, &P < 0.05 to sham.

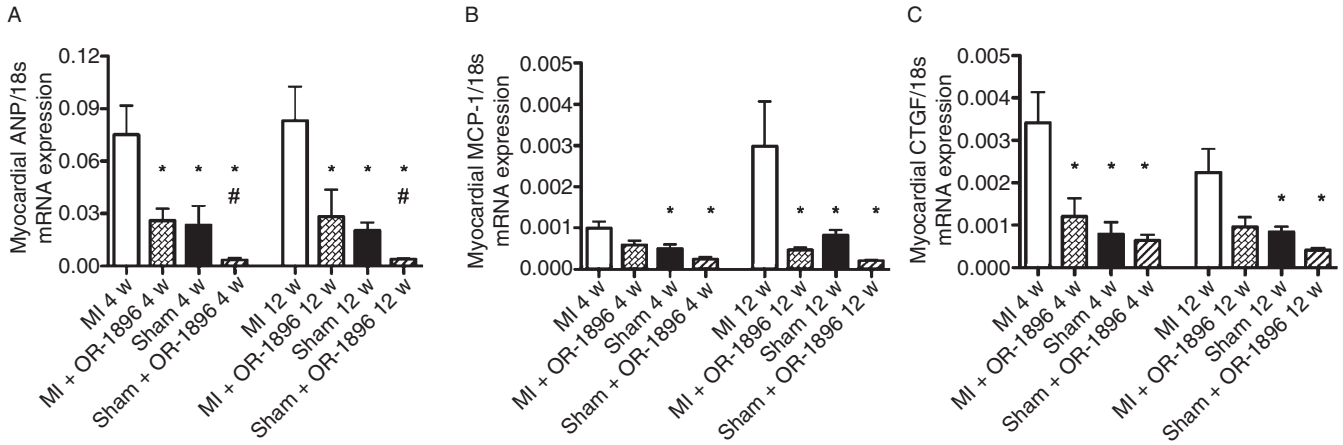

Figure 3 Effects of oral OR-1896 treatment on myocardial mRNA expressions of ANP (A), MCP-1 (B) and CTGF (C) in spontaneously diabetic GK rats 4 and 12 weeks after MI or sham operation. Abbreviations for experimental groups as in Figure 1. Means ± SEM are given, $n = 6–12$ in each group. *$P < 0.05$ when compared to age-matched GK MI.

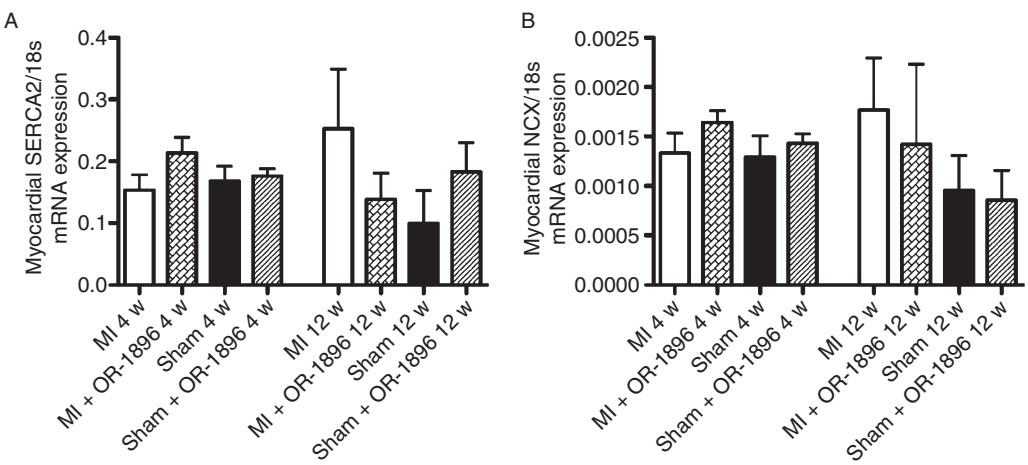

Figure 4 Effects of oral OR-1896 treatment on mRNA expression of calcium handling proteins, SERCA2 and NCX, in spontaneously diabetic GK rats 4 and 12 weeks after MI or sham operation. Abbreviations for experimental groups as in Figure 1. Means ± SEM are given, $n = 6–12$ in each group.

Cardiovascular effects of vasodilation by valsartan in GK rats with MI

In GK rats with MI, valsartan decreased blood pressure by 30 mm Hg (systolic blood pressure 93 ± 1 vs. 124 ± 2 mm Hg, $P < 0.0001$). However, 4 weeks of treatment with valsartan did not influence cardiac function measured as ejection fraction (33.9 ± 6.8 vs. 22.7 ± 2.7%, $P = 0.32$) or fractional shortening (20.1 ± 6.0 vs. 9.4 ± 2.3%, $P = 0.095$). Valsartan-induced decrease in blood pressure was associated with amelioration of post-infarct cardiac hypertrophy (heart weight-to-body weight ratio 3.9 ± 0.2 vs. 5.6 ± 0.3 $mg \cdot g^{-1}$, $P = 0.011$). In GK rats with MI, valsartan reduced cardiac MCP-1 (MCP1-to-18S mRNA ratio 0.34 ± 0.07 vs. 0.14 ± 0.02, $P = 0.04$) and CTGF (CTGF-to-18S mRNA ratio 0.12 ± 0.02 vs. 0.038 ± 0.003, $P = 0.002$) mRNA expressions, but did not influence cardiac ANP mRNA expression (ANP-to-18S mRNA ratio ($P = 0.09$) or expression of $p16^{INK4A}$ ($P = 0.32$).

Biochemical analyses

Data from biochemical and hormonal analyses are presented in Table 3. Neither MI nor OR-1896 treatment influenced PRA, blood glucose or serum insulin. The average terminal plasma concentration of OR-1896 in OR-1896-treated rats was 20.1 ± 2.0 $ng \cdot mL^{-1}$.

Discussion

Levosimendan is a calcium sensitizer used primarily in the management of acute heart failure, and more recently also in patients with ischaemic heart disease and cardiogenic or septic shock (Nieminen *et al.*, 2009). The long-term effects of calcium sensitizers are still largely unknown at the moment. The aim of this study was to investigate the cardiovascular effects of orally administered OR-1896 in experimental heart failure. Using LAD ligation and spontaneously diabetic GK rats as model of type 2 diabetic post-infarct heart failure, we here demonstrated that OR-1896 improved systolic function after MI, and effectively ameliorated post-infarct cardiomyocyte hypertrophy and cardiac remodelling.

Levosimendan is currently used only as a 24 h systemic infusion to improve the symptoms of acute decompensated

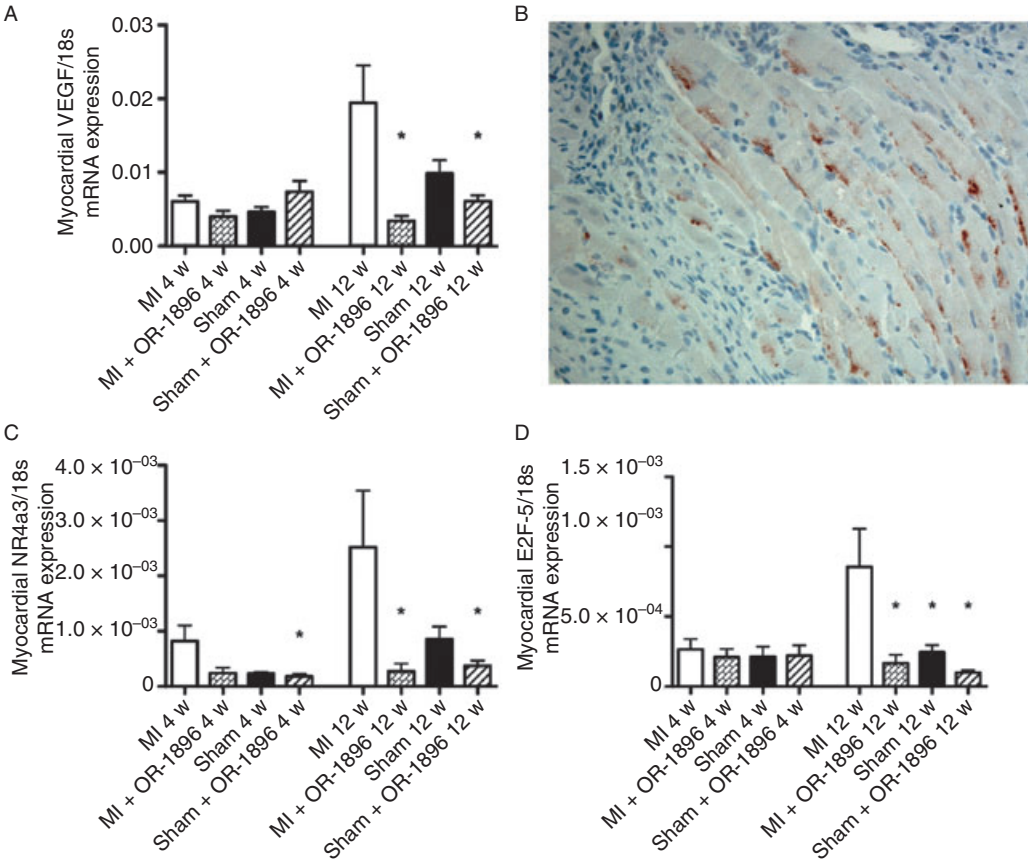

Figure 5 Effects of oral OR-1896 treatment on myocardial mRNA expressions of VEGF (A), NR4a3 (C) and E2F-5 (D) in spontaneously diabetic GK rats 4 and 12 weeks after MI or sham operation. (B) A section of GK rat heart with MI, stained for c-kit. Abbreviations for experimental groups as in Figure 1. Means ± SEM are given, $n = 6$–12 in each group. *$P < 0.05$ when compared to age-matched GK MI.

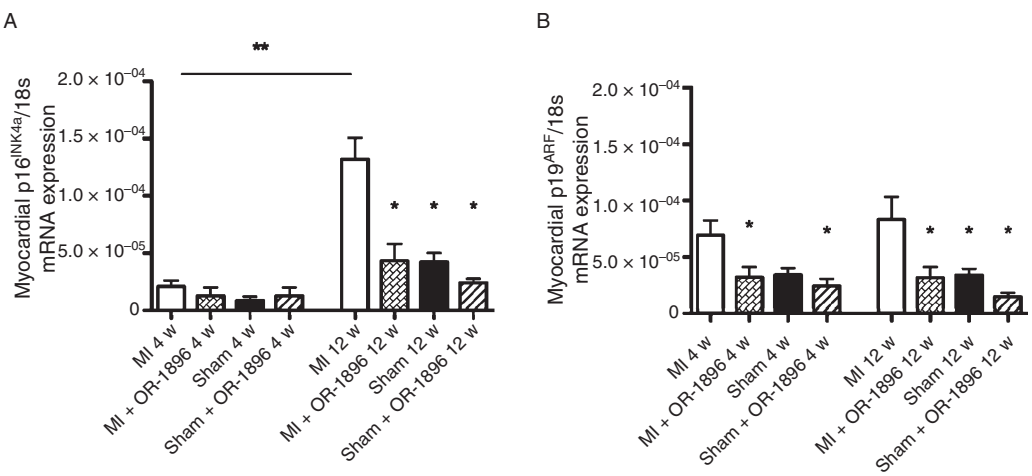

Figure 6 Effects of oral OR-1896 treatment on myocardial senescence markers $p16^{INK4A}$ (A) and $p19^{ARF}$ (B) in spontaneously diabetic GK rats 4 and 12 weeks after MI or sham operation. Abbreviations for experimental groups as in Figure 1. Means ± SEM are given, $n = 6$–12 in each group. *$P < 0.05$ when compared to age-matched MI, **$P < 0.05$ when compared MI 4 weeks to MI 12 weeks.

heart failure. In a very recent PERSIST study, the effects of oral levosimendan treatment were investigated in patients suffering from severe chronic heart failure (Nieminen *et al.*, 2008). Although oral levosimendan did not decrease cardiovascular mortality, an improvement in quality of life and a persistent reduction in plasma brain natriuretic peptide level were reported (Nieminen *et al.*, 2008). We have shown recently that oral levosimendan treatment improves survival and ameliorates hypertension-induced cardiac remodelling in Dahl salt-sensitive rats (Louhelainen *et al.*, 2007), and prevents

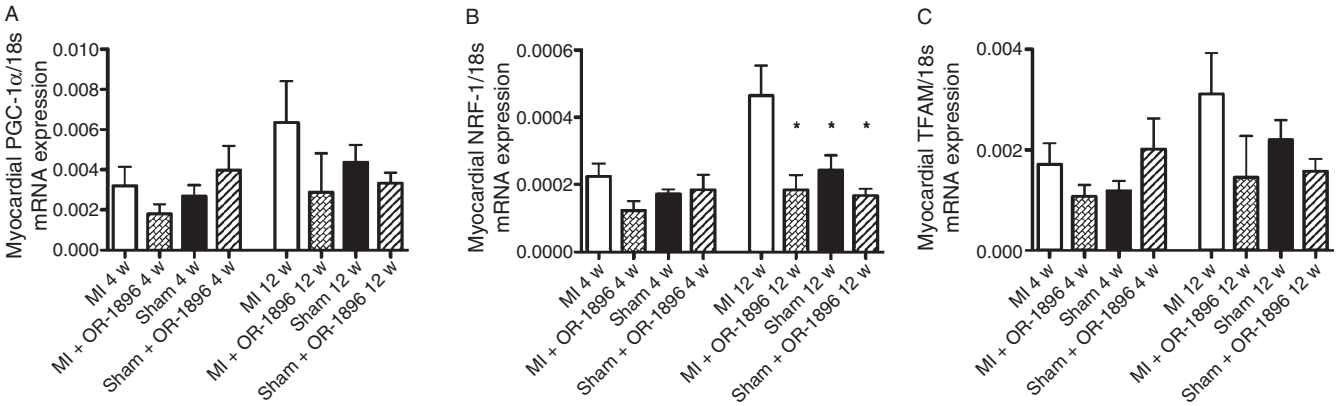

Figure 7 Effects of oral OR-1896 treatment on mRNA expression of mitochondrial biogenesis markers PGC-1 (A) NRF-1 (B) and TFAM (C) in spontaneously diabetic GK rats 4 and 12 weeks after MI or sham operation. Abbreviations for experimental groups as in Figure 1. Means ± SEM are given, n = 6–12 in each group. *$P < 0.05$ when compared to age-matched MI.

Table 3 Biochemical and hormonal parameters in spontaneously diabetic GK rats 4 and 12 weeks after MI

Variable	*GK MI 4 weeks*	*GK MI + OR-1896 4 weeks*	*GK sham 4 weeks*	*GK sham + OR-1896 4 weeks*	*anova P value*
Blood glucose, mmol·L^{-1}	6.5 ± 0.5	7.8 ± 0.5	7.5 ± 0.3	8.9 ± 0.9	0.041
Serum insulin, ng·mL^{-1}	2.7 ± 0.3	2.1 ± 0.2	3.1 ± 0.3	2.4 ± 0.4	0.125
PRA, ng·mL^{-1}·h^{-1}	2.3 ± 0.3	2.3 ± 0.3	3.4 ± 0.6	2.2 ± 0.4	0.190
Variable	*GK MI 12 weeks*	*GK MI + OR-1896 12 weeks*	*GK sham 12 weeks*	*GK sham + OR-1896 12 weeks*	*anova P value*
Blood glucose, mmol·L^{-1}	8.2 ± 0.7	7.8 ± 0.5	8.4 ± 0.9	8.3 ± 0.5	0.090
Serum insulin, ng·mL^{-1}	3.3 ± 0.6	2.9 ± 0.3	2.2 ± 0.2	1.9 ± 1.2	0.077
PRA, ng·mL^{-1}·h^{-1}	1.9 ± 0.2	2.4 ± 0.4	2.1 ± 0.5	1.7 ± 0.3	0.554

OR-1896 was given orally at a daily dose of 0.5 mg·kg^{-1}. Data in the table are means ± SEM, n = 5–11 in each group.

post-infarct heart failure in spontaneously diabetic GK rats (Louhelainen *et al.*, 2009). It is believed that the cardiovascular effects of chronic levosimendan treatment are mediated to a great extent by the pharmacologically active and long-lasting metabolite OR-1896. Therefore, we tested the hypothesis whether beneficial cardiovascular effects, similar to those of the parent compound, could be achieved by oral treatment with OR-1896 alone. The dosage of OR-1896 used in the present study was selected to produce a plasma OR-1896 concentration, comparable to those reported after oral and intravenous levosimendan treatments (Antila *et al.*, 2007; Louhelainen *et al.*, 2007; 2009).

In the present study, oral OR-1896 improved systolic function after MI and post-infarct cardiac remodelling assessed as cardiomyocyte hypertrophy, as well as gene markers of cardiac pressure/volume overload, inflammation, fibrosis and cellular senescence. These findings indicate that OR-1896-induced calcium sensitization and opening of the ATP-sensitive potassium channels display distinct time profiles in our animal model. Surprisingly, OR-1896 did not decrease cardiovascular mortality. However, it should be emphasized that the cardiovascular mortality in our experimental diabetic myocardial infarct model occurred also almost completely during the first 24 h after the operation, whereas OR-1896 treatment was started 24 h after operation. In the present study, the follow-up time lasted only until 12 weeks, and we noticed only minor cardiovascular mortality in GK rats with MI during this time period. Hence, the short duration of our study could also explain, at least in part, the lack of any effect by OR-1896 on cardiovascular mortality after MI. Consistent with our present results, levosimendan did not influence cardiovascular mortality or myocardial infarct size in GK rats when given 24 h after MI (Louhelainen *et al.*, 2009).

Premature senescence has been linked to several pathological conditions, such as MI and type 2 diabetes (Sharpless and DePinho, 2007; Melzer, 2008) Activation of the senescence marker p16^{INK4A} has recently been shown to be linked to hypertensive heart disease (Westhoff *et al.*, 2008) and to diabetes-induced excess reactive oxygen species production (Rota *et al.*, 2006). In good accordance with previous findings, MI induced clear, age-dependent senescent response in GK rats. In the present study, we showed that despite of being encoded in same locus, p19^{ARF} and p16^{INK4A} show strikingly different expression profiles during post-infarct cardiac remodelling, indicating separate roles in response to cardiac trauma and ageing. Myocardial expression of p19^{ARF} mRNA was induced immediately after MI, with most prominent expression at early weeks of post-infarct remodelling. On the contrary, p16^{INK4A} mRNA expression was induced by several folds at later stages of post-infarct cardiac remodelling.

Interestingly, Baker *et al*. (2008) very recently reported the attenuating role of $p19^{ARF}$ in senescence and ageing, indicating that $p19^{ARF}$ functions as a protective factor to alleviate MI-induced trauma. Based on the later activation of $p16^{INK4A}$ mRNA expression during post-infarct cardiac remodelling, it appears that $p16^{INK4A}$ is activated rather by diabetes-aggravated ageing than by MI alone. Similarly, MI induced a significant rise in expression of transcription factor E2F-5, which has been shown to be a key downstream mediator for $p16^{INK4A}$-induced G1 arrest (Gaubatz *et al.*, 2000; Ohtani *et al.*, 2003). Interestingly, oral OR-1896 treatment was able to completely normalize mRNA expression of both $p19^{ARF}$ and $p16^{INK4A}$, and also E2F-5. The mechanism of this suppression of senescence signalling is unclear, but it could have been mediated via reduced oxidative stress. MI resulted in a significant increase in the mRNA expression of VEGF and NR4a3 (Liu *et al.*, 2003; Martinez-Gonzalez and Badimon, 2005). Interestingly, NR4a3 has been shown to control MCP-1 expression, and modulate inflammatory responses, which could explain the reduced senescence signalling as a response to improved inflammatory status (Pols *et al.*, 2007).

Accumulating evidence suggests that congestive heart failure is associated with alterations in energy metabolism and mitochondrial function. Garnier *et al.*, (2003) reported recently depressed mitochondrial transcription factors and oxidative capacity in the failing rat heart. To explore mitochondrial biogenesis in post-infarct heart failure, we analysed the mRNA expressions of transcription factors such as TFAM, PGC-1α and NRF-1 in the heart. Interestingly, we found a statistically significant increase in the expression of myocardial NRF-1 12 weeks after MI. We also noticed a non-significant increase both in PGC-1α and TFAM mRNA expressions in diabetic post-infarct rat heart. In line with our findings, Sano and Fukuda, (2008) introduced very recently a novel concept that mitochondrial biogenesis can in fact be triggered by low levels of reactive oxygen species. Treatment with OR-1896 prevented the MI-induced increase in mitochondrial biogenesis markers, thus supporting the notion that OR-1896 may exert anti-inflammatory and/or antioxidative properties.

Ejection fraction and fractional shortening are highly dependent on afterload and systemic blood pressure. As improvement of heart function and restoration of MI-induced alterations in cardiac gene expression profile could thus be attributed to drug-induced decrease in blood pressure and overall haemodynamics, we examined in a separate experimental study the cardiovascular effects of the angiotensin receptor antagonist valsartan, a widely used antihypertensive agent, in GK rats with MI. We were able to demonstrate, that valsartan decreased systolic blood pressure in GK rats with MI by approximately 30 mm Hg. Valsartan-induced decrease in blood pressure was associated with amelioration of cardiac hypertrophy. However, valsartan treatment did not significantly improve cardiac function, measured as ejection fraction or fractional shortening. Our findings thus suggest that the beneficial cardiac effects of OR-1896 cannot be explained solely by drug-induced decrease in systemic blood pressure and peripheral vascular resistance. It should also be emphasized that in the present study, OR-1896 did not decrease blood pressure in GK rats with MI, although it clearly decreased blood pressure in sham-operated GK rats.

Type II diabetes predisposes to ischaemic heart disease, and development of MI and heart failure. We showed previously that diabetes-induced cardiac remodelling in GK rats was associated with cardiac hypertrophy, systolic dysfunction and increased apoptotic signalling (Vahtola *et al.*, 2008). Furthermore, we have demonstrated very recently (Louhelainen *et al.*, 2009) that levosimendan prevented post-infarct heart failure and cardiac remodelling in diabetic GK rats, whereas the beneficial effects of levosimendan were less pronounced in non-diabetic Wistar rats after MI. Therefore, we selected diabetic GK rats as a model of experimental heart failure for the present study. Spontaneously diabetic GK rats exhibit salt-sensitive hypertension and endothelial dysfunction (Galli *et al.*, 1999; Cheng *et al.*, 2001). We have shown, using direct blood pressure measurement with radiotelemetry, that systolic blood pressure in GK rats is slightly increased even when kept on normal sodium diet as compared with their non-diabetic Wistar controls (Cheng *et al.*, 2001). The important finding of the study was that after the initial elevation of blood pressure due to increased cardiac output, OR-1896 decreased blood pressure in sham-operated GK rats. The blood pressure-lowering effect of OR-1896 was associated with amelioration of cardiac hypertrophy, whereas cardiac functions measured by echocardiography remained unaltered by OR-1896 at weeks 4 and 12. The beneficial effect of OR-1896 on cardiac hypertrophy was likely to have been mediated by vasodilatation, and thus, decreased pressure load imposed to the heart. Decreased myocardial ANP mRNA expression in OR-1896-treated GK rats strongly supports this notion.

The present study has some limitations. Relatively small sample size in each study group and testing of multiple study groups render the present study susceptible to hyperinflation error. As ejection fraction and fractional shortening are highly dependent on systemic blood pressure, the present study cannot distinguish whether the primary mechanism of action leading to improvement of cardiac function and tissue protection after MI was due to OR-1896-induced vasodilation or drug-induced positive inotropic effect. Measurement of dP/dt or pressure–volume relationship would have been highly valuable in clarifying the point. Finally, our study design did not include echocardiography measurements after operations and before randomization of the animals into the different treatment groups. Therefore, further studies are warranted to examine in detail the influence of OR-1896 on MI-induced cardiac dysfunction during the first study week after the operation.

In conclusion, classic positive inotropic agents provide short-term haemodynamic improvement in patients with heart failure, but their long-term use has been associated with poor prognosis. OR-1896, the active and long-lasting metabolite of levosimendan, exerts positive inotropic action via calcium sensitization and a vasodilatory effect via activation of the ATP-sensitive K^+-channel. We here demonstrated that oral treatment with OR-1896 prevents post-infarct heart failure and cardiac remodelling in spontaneously diabetic GK rats. The present study thus suggests a therapeutic role for oral OR-1896 in the prevention of post-infarct heart failure and cardiac remodelling in type 2 diabetes.

Acknowledgements

This study was supported by grants from the Academy of Finland, Sigrid Jusélius Foundation, Päivikki ja Sakari Sohlberg Foundation, Finnish Foundation of Cardiovascular Research and Orion-Farmos Research Foundation. We are grateful to Ms Anneli von Behr, Ms Sari Laakkonen and Ms Essi Martonen for expert technical assistance. OR-1896 was a generous gift from Orion Pharma.

Conflict of interest

None.

References

Antila S, Sundberg S, Lehtonen LA (2007). Clinical pharmacology of levosimendan. *Clin Pharmacokinet* **46**: 535–552.

Baker DJ, Perez-Terzic C, Jin F, Pitel K, Niederlander NJ, Jeganathan K *et al.* (2008). Opposing roles for p16Ink4a and p19Arf in senescence and ageing caused by BubR1 insufficiency. *Nat Cell Biol* **10**: 825–836.

Banfor PN, Preusser LC, Campbell TJ, Marsh KC, Polakowski JS, Reinhart GA *et al.* (2008). Comparative effects of levosimendan, OR-1896, OR-1855, dobutamine, and milrinone on vascular resistance, indexes of cardiac function, and O_2 consumption in dogs. *Am J Physiol Heart Circ Physiol* **294**: H238–H248.

Bastide K, Guilly MN, Bernaudin JF, Joubert C, Lectard B, Levalois C *et al.* (2008). Molecular analysis of the Ink4a/Rb1-Arf/Tp53 pathways in radon-induced rat lung tumors. *Lung Cancer* **63**: 348–353.

Bowman P, Haikala H, Paul RJ (1999). Levosimendan, a calcium sensitizer in cardiac muscle, induces relaxation in coronary smooth muscle through calcium desensitization. *J Pharmacol Exp Ther* **288**: 316–325.

Cheng ZJ, Vaskonen T, Tikkanen I, Nurminen K, Ruskoaho H, Vapaatalo H *et al.* (2001). Endothelial dysfunction and salt-sensitive hypertension in spontaneously diabetic Goto–Kakizaki rats. *Hypertension* **37**: 433–439.

Endoh M (2008). Cardiac Ca^{2+} signaling and Ca^{2+} sensitizers. *Circ J* **72**: 1915–1925.

Erdei N, Papp Z, Pollesello P, Edes I, Bagi Z (2006). The levosimendan metabolite OR-1896 elicits vasodilation by activating the K(ATP) and BK(ca) channels in rat isolated arterioles. *Br J Pharmacol* **148**: 696–702.

Galli J, Fakhrai-Rad H, Kamel A, Marcus C, Norgren S, Luthman H (1999). Pathophysiological and genetic characterization of the major diabetes locus in GK rats. *Diabetes* **48**: 2463–2470.

Garnier A, Fortin D, Delomenie C, Momken I, Veksler V, Ventura-Clapier R (2003). Depressed mitochondrial transcription factors and oxidative capacity in rat failing cardiac and skeletal muscles. *J Physiol* **551**: 491–501.

Gaubatz S, Lindeman GJ, Ishida S, Jakoi L, Nevins JR, Livingston DM *et al.* (2000). E2F4 and E2F5 play an essential role in pocket protein-mediated G1 control. *Mol Cell* **6**: 729–735.

Haikala H, Nissinen E, Etemadzadeh E, Levijoki J, Linden IB (1995). Troponin C-mediated calcium sensitization induced by levosimendan does not impair relaxation. *J Cardiovasc Pharmacol* **25**: 794–801.

Heyen JR, Blasi ER, Nikula K, Rocha R, Daust HA, Frierdich G *et al.* (2002). Structural, functional, and molecular characterization of the SHHF model of heart failure. *Am J Physiol Heart Circ Physiol* **283**: H1775–H1784.

Innes CA, Wagstaff AJ (2003). Levosimendan: a review of its use in the management of acute decompensated heart failure. *Drugs* **63**: 2651–2671.

Kivikko M, Lehtonen L, Colucci WS (2003). Sustained hemodynamic effects of intravenous levosimendan. *Circulation* **107**: 81–86.

Kopustinskiene DM, Pollesello P, Saris NE (2004). Potassium-specific effects of levosimendan on heart mitochondria. *Biochem Pharmacol* **68**: 807–812.

Levijoki J, Pollesello P, Kaheinen P, Haikala H (2001). Improved survival with simendan after experimental myocardial infarction in rats. *Eur J Pharmacol* **419**: 243–248.

Liu D, Jia H, Holmes DI, Stannard A, Zachary I (2003). Vascular endothelial growth factor-regulated gene expression in endothelial cells: KDR-mediated induction of Egr3 and the related nuclear receptors Nur77, Nurr1, and Nor1. *Arterioscler Thromb Vasc Biol* **23**: 2002–2007.

Louhelainen M, Vahtola E, Kaheinen P, Leskinen H, Merasto S, Kyto V *et al.* (2007). Effects of levosimendan on cardiac remodeling and cardiomyocyte apoptosis in hypertensive Dahl/Rapp rats. *Br J Pharmacol* **150**: 851–861.

Louhelainen M, Vahtola E, Forsten H, Merasto S, Kyto V, Finckenberg P *et al.* (2009). Oral levosimendan prevents postinfarct heart failure and cardiac remodeling in diabetic Goto–Kakizaki rats. *J Hypertens* **27**: 2094–2107.

Martinez-Gonzalez J, Badimon L (2005). The NR4A subfamily of nuclear receptors: new early genes regulated by growth factors in vascular cells. *Cardiovasc Res* **65**: 609–618.

Melzer D (2008). Genetic polymorphisms and human aging: association studies deliver. *Rejuvenation Res* **11**: 523–526.

Nieminen MS, Cleland JG, Eha J, Belenkov Y, Kivikko M, Poder P *et al.* (2008). Oral levosimendan in patients with severe chronic heart failure – the PERSIST study. *Eur J Heart Fail* **10**: 1246–1254.

Nieminen MS, Pollesello P, Vajda G, Papp Z (2009). Effects of levosimendan on the energy balance: preclinical and clinical evidence. *J Cardiovasc Pharmacol* **53**: 302–310.

Ohtani N, Brennan P, Gaubatz S, Sanij E, Hertzog P, Wolvetang E *et al.* (2003). Epstein-barr virus LMP1 blocks p16INK4a-RB pathway by promoting nuclear export of E2F4/5. *J Cell Biol* **162**: 173–183.

Ozdem SS, Yalcin O, Meiselman HJ, Baskurt OK, Usta C (2006). The role of potassium channels in relaxant effect of levosimendan in rat small mesenteric arteries. *Cardiovasc Drugs Ther* **20**: 123–127.

Palojoki E, Saraste A, Eriksson A, Pulkki K, Kallajoki M, Voipio-Pulkki LM *et al.* (2001a). Cardiomyocyte apoptosis and ventricular remodeling after myocardial infarction in rats. *Am J Physiol Heart Circ Physiol* **280**: H2726–H2731.

Palojoki E, Saraste A, Eriksson A, Pulkki K, Kallajoki M, Voipio-Pulkki LM *et al.* (2001b). Cardiomyocyte apoptosis and ventricular remodeling after myocardial infarction in rats. *Am J Physiol Heart Circ Physiol* **280**: H2726–H2731.

Pfeffer MA, Pfeffer JM, Fishbein MC, Fletcher PJ, Spadaro J, Kloner RA *et al.* (1979). Myocardial infarct size and ventricular function in rats. *Circ Res* **44**: 503–512.

Pols TW, Bonta PI, de Vries CJ (2007). NR4A nuclear orphan receptors: protective in vascular disease? *Curr Opin Lipidol* **18**: 515–520.

Rota M, LeCapitaine N, Hosoda T, Boni A, De Angelis A, Padin-Iruegas ME *et al.* (2006). Diabetes promotes cardiac stem cell aging and heart failure, which are prevented by deletion of the *p66shc* gene. *Circ Res* **99**: 42–52.

Sandell EP, Hayha M, Antila S, Heikkinen P, Ottoila P, Lehtonen LA *et al.* (1995). Pharmacokinetics of levosimendan in healthy volunteers and patients with congestive heart failure. *J Cardiovasc Pharmacol* **26** (Suppl 1): S57–S62.

Sano M, Fukuda K (2008). Activation of mitochondrial biogenesis by hormesis. *Circ Res* **103**: 1191–1193.

Segreti JA, Marsh KC, Polakowski JS, Fryer RM (2008). Evoked changes in cardiovascular function in rats by infusion of levosimendan, OR-1896 [(*R*)-*N*-(4-(4-methyl-6-oxo-1,4,5,6-tetrahydropyridazin-3-

yl)phenyl)acetamide], OR-1855 [(*R*)-6-(4-aminophenyl)-5-methyl-4,5-dihydropyridazin-3(2H)-one], dobutamine, and milrinone: comparative effects on peripheral resistance, cardiac output, d*P*/d*t*, pulse rate, and blood pressure. *J Pharmacol Exp Ther* **325**: 331–340.

Sharpless NE, DePinho RA (2007). How stem cells age and why this makes us grow old. *Nat Rev Mol Cell Biol* **8**: 703–713.

Sorsa T, Heikkinen S, Abbott MB, Abusamhadneh E, Laakso T, Tilgmann C *et al*. (2001). Binding of levosimendan, a calcium sensitizer, to cardiac troponin C. *J Biol Chem* **276**: 9337–9343.

Szilagyi S, Pollesello P, Levijoki J, Kaheinen P, Haikala H, Edes I *et al*. (2004). The effects of levosimendan and OR-1896 on isolated hearts, myocyte-sized preparations and phosphodiesterase enzymes of the guinea pig. *Eur J Pharmacol* **486**: 67–74.

Takahashi R, Endoh M (2002). Effects of OR-1896, a metabolite of levosimendan, on force of contraction and Ca^{2+} transients under acidotic condition in aequorin-loaded canine ventricular myocardium. *Naunyn Schmiedebergs Arch Pharmacol* **366**: 440–448.

Takahashi R, Talukder MA, Endoh M (2000). Inotropic effects of OR-1896, an active metabolite of levosimendan, on canine ventricular myocardium. *Eur J Pharmacol* **400**: 103–112.

du Toit EF, Genis A, Opie LH, Pollesello P, Lochner A (2008). A role for the RISK pathway and K(ATP) channels in pre- and post-conditioning induced by levosimendan in the isolated guinea pig heart. *Br J Pharmacol* **154**: 41–50.

Vahtola E, Louhelainen M, Merasto S, Martonen E, Penttinen S, Aahos I *et al*. (2008). Forkhead class O transcription factor 3a activation and sirtuin1 overexpression in the hypertrophied myocardium of the diabetic Goto–Kakizaki rat. *J Hypertens* **26**: 334–344.

Wellner M, Dechend R, Park JK, Shagdarsuren E, Al-Saadi N, Kirsch T *et al*. (2005). Cardiac gene expression profile in rats with terminal heart failure and cachexia. *Physiol Genomics* **20**: 256–267.

Westhoff JH, Hilgers KF, Steinbach MP, Hartner A, Klanke B, Amann K *et al*. (2008). Hypertension induces somatic cellular senescence in rats and humans by induction of cell cycle inhibitor p16INK4a. *Hypertension* **52**: 123–129.