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# Vasopeptidase inhibition has beneficial cardiac effects in spontaneously diabetic Goto–Kakizaki rats

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**Abstract**

In this study we examined diabetes- and hypertension-induced changes in cardiac structure and function in an animal model of type 2 diabetes, the Goto–Kakizaki (GK) rat. We hypothesized that treatment with omapatrilat, a vasopeptidase inhibitor, which causes simultaneous inhibition of angiotensin converting enzyme and neutral endopeptidase, provides additional cardioprotective effects, during normal as well as high sodium intake, compared to treatment with enalapril, a selective inhibitor of angiotensin converting enzyme. Fifty-two GK rats were randomized into 6 groups to receive either normal-sodium (NaCl 0.8%) or high-sodium (NaCl 6%) diet and enalapril, omapatrilat or vehicle for 12 weeks. The GK rats developed hypertension, cardiac hypertrophy and overexpression of cardiac natriuretic peptides and profibrotic connective tissue growth factor compared to nondiabetic Wistar rats. The high dietary sodium further increased the systolic blood pressure, and changed the mitral inflow pattern measured by echocardiography towards diastolic dysfunction. Enalapril and omapatrilat equally decreased the systolic blood pressure compared to the control group during normal- as well as high-sodium diet. Both drugs had beneficial cardioprotective effects, which were blunted by the high dietary sodium. Compared to enalapril, omapatrilat reduced the echocardiographically measured left ventricular mass during normal-sodium diet and improved the diastolic function during high-sodium diet in GK rats. Furthermore, omapatrilat reduced relative cardiac weight more effectively than enalapril during high sodium intake. Our results suggest that both the renin–angiotensin and the neutral endopeptidase system are involved in the pathogenesis of diabetic cardiomyopathy since vasopeptidase inhibition was shown to provide additional benefits in comparison with selective angiotensin converting enzyme inhibition alone.


*Keywords:* Hypertension; Diabetes; Angiotensin converting enzyme inhibition; Vasopeptidase inhibition; Cardiomyopathy

## 1. Introduction

Both type 1 and type 2 diabetes have been shown to be independent risk factors for left ventricular dysfunction and heart failure (Ye et al., 2004; Tshöpe et al., 2004). Diabetic cardiomyopathy is characterized by impairment in cardiac diastolic performance, which precedes systolic dysfunction (Uusitupa et al., 1990; Mizushige et al., 2000; Cosson and Kevorkian, 2003). The exact mechanisms leading to diabetic heart disease are still unknown, but several factors such as hyperglycemia, insulin resistance, damage caused by free radicals and enhanced tissue renin–angiotensin system activity are

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supposed to contribute to the pathogenesis (Privratsky et al., 2003).

The coexisting hypertension accounts for up to 75% of added cardiovascular disease risk in diabetics and contributes decisively to the morbidity and mortality in these high-risk patients (El-Atat et al., 2004). Previous studies have shown that aggressive blood pressure control is effective in cardiovascular disease reduction in hypertensive diabetic subjects (Laakso, 2001). Still, the target blood pressure is difficult to achieve in the majority of these patients. The blockade of renin–angiotensin system with angiotensin converting enzyme inhibitors has been proved, at least partly, to prevent the development of cardiovascular complications of type 2 diabetes and hypertensive disease (Ruilope et al., 2002). However, a common problem in clinical practice is the high salt intake, which blunts the blood pressure-lowering effect of renin–angiotensin system inhibition.

The vasopeptidase inhibitors have been developed to improve the efficacy and benefits of angiotensin converting enzyme inhibition. The vasopeptidase inhibitors inhibit simultaneously both angiotensin converting enzyme and neutral endopeptidase. The neutral endopeptidase is involved in the metabolism of several vasoactive peptides including A-, B-, and C-type natriuretic peptides (ANP, BNP and CNP), kinins, (bradykinin and kallidin), adrenomedullin, angiotensin II and endothelin.

The vasopeptidase inhibitor omapatrilat has been demonstrated to effectively decrease blood pressure in different models of hypertension independently of renin- or salt-status (Tikkanen et al., 1998; Trippodo et al., 1998; Burnett, 1999; Burrell et al., 2000). Furthermore, omapatrilat has been shown to have beneficial hemodynamic and renal effects in experimental heart failure (Troughton et al., 2000; Cataliotti et al., 2002; Campbell, 2003) and to have promising vasculoprotective properties in salt-induced hypertension (Quaschning et al., 2001; Millette et al., 2003). Thus, dual angiotensin converting enzyme and neutral endopeptidase inhibition could provide additional cardioprotective effects from angiotensin converting enzyme inhibition alone in diabetes-associated hypertension and cardiomyopathy.

The processes leading to diabetic cardiomyopathy have mostly been investigated in animal models of insulin-deficient diabetes and there are only few studies in animal models of type 2 diabetes. To investigate the alterations in myocardial function in type 2 diabetes, we used the Goto–Kakizaki (GK) rat, a non-obese rat model of type 2 diabetes originally derived by repeated inbreeding of glucose-intolerant Wistar rats (Goto et al., 1976). The GK rat is characterized by the development of a polygenic, spontaneous type 2 diabetes, insulin resistance and abnormal glucose metabolism (Galli et al., 1999; Janssen et al., 2003). We have previously demonstrated the involvement of renin–angiotensin system in the pathogenesis of salt-sensitive hypertension, endothelial dysfunction and diabetic nephropathy in GK rats (Cheng et al., 2001). As the same mechanisms may be implicated also in cardiac disease, we now examined the effectiveness of an angiotensin converting enzyme inhibitor, enalapril, as compared with a vasopeptidase inhibitor, omapatrilat, in preventing the development of hypertension- and diabetes-induced changes in cardiac structure and function in GK rats. To our best knowledge, in vivo cardiac function assessed by echocardiography has not earlier been described in the GK rat.

## 2. Materials and methods

### 2.1. Experimental animals, drug regimen and sample preparation

Fifty-two 8-week-old male GK rats (M and B, Ejby, Denmark) and ten age-matched Wistar rats were housed at 23–25 °C in a 12-h light/dark cycle with free access to rat chow and normal drinking water. The experimental procedures were approved by the Animal Experimentation Committee of University of Helsinki, Helsinki, Finland.

Blood pressure- and body weight-matched GK rats were divided into 6 groups ($n$=8–9 in each group) to receive different diet and drug regimens for 12 weeks: 1) normal-sodium diet controls (NaCl 0.8%); 2) normal-sodium diet+enalapril (30 mg/kg/d); 3) normal-sodium diet+omapatrilat (40 mg/kg/d); 4) high-sodium diet controls (NaCl 6%); 5) high-sodium diet+enalapril; and 6) high-sodium diet+omapatrilat. The Wistar rats on a normal-sodium diet served as nondiabetic controls. The special research diets were prepared by mixing into the powdered rat chow (2018S, Harlan Teklad Global Diets, Harlan Holding Inc., Wilmington, DE, USA) the chosen amount of NaCl (wt/wt) and the powdered drug.

The drug doses were chosen on basis of the results of earlier studies of our research group (Bäcklund et al., 2001, 2003; Grönholm et al., 2004) and other previously published experimental works (Trippodo et al., 1998; Nunez et al., 1997), targeting at equal blood pressure-lowering effect of enalapril and omapatrilat during normal-sodium diet. The actual drug doses were calculated by measuring the daily food intake in metabolic cages before the treatment period started, and adjusted during the study period by remeasuring the food consumption every fourth week. Omapatrilat was provided as a gift from Bristol-Myers-Squibb Pharmaceuticals (Princeton, NY) and enalapril from Leiras (Helsinki, Finland).

Systolic blood pressure was measured at 4 weeks intervals in conscious lightly restrained rats by a tail-cuff blood pressure analyzer (Apollo-2AB Blood Pressure Analyzer, model 179-2AB, IITC Life Science, Woodland Hills, CA, USA). At each time the systolic blood pressure was assessed from an average of 3–5 measurements for each rat.

At the end of the experiment the rats were anesthetized by intraperitoneal administration of pentobarbital (55 mg/kg; Mebunat vet®, Orion Pharma, Espoo, Finland). Blood samples were collected into pre-chilled lithium heparin tubes and into tubes containing $Na_2EDTA$ (150 mmol/l) and aprotinin (500 kallikrein inhibitor units per milliliter). The heart was excised, washed with ice-cold saline, blotted dry, and weighed. Two 2 mm thick transverse sections from the midventricular level were fixed in 4% neutral buffered paraformaldehyde overnight and then

processed in paraffin with routine techniques for further analysis. Samples for immunohistochemistry were snap-frozen in isopentane (−35 °C) and the remaining apical and basal parts were snap-frozen in liquid nitrogen.

### 2.2. Echocardiography

At the end of the treatment period cardiac function was determined by two-dimensional echocardiography using a 7–10 MHz phased array sector probe (GE VingMed Vivid FiVe Echocardiography System, General Electric Company, UK) from the right parasternal projection. Prior to echocardiography the animals were sedated by medetomidine (0.25 mg/kg s.c.; Domitor®, Orion Pharma, Espoo, Finland). The sedative effect of medetomidine was eliminated by atipamezole 0.75 mg/kg s.c. (Antisedan®, 5 mg/ml, Orion Pharma, Espoo, Finland) after the echocardiographic measurements. Normothermia was maintained by placing the sedated animals on a thermal plate. An average of three measurements per each animal was used to assess intraventricular septum and posterior wall thickness as well as left ventricular end diastolic diameter and left ventricular end systolic diameter. Left ventricular end diastolic volume was measured by Simpson's formula. The left ventricular mass was calculated from a standard cube formula (Litwin et al., 1994; Bäcklund et al., 2003). The left ventricular fractional shortening was determined by the equation: fractional shortening = ((left ventricular end diastolic diameter − left ventricular end systolic diameter) / left ventricular end diastolic diameter) × 100%. Transmitral flow waveforms were recorded using pulsed wave Doppler oriented in the parasternal long axis. Peak transmitral (E wave) flow, E wave deceleration slope and A wave flow were determined and used as measures of diastolic ventricular function. The echocardiographic parameters were analyzed in a blinded fashion.

### 2.3. Myocardial fibrosis

Collagen volume fraction was determined by the picrosirius red method from three different slices of Direct Red 80 (Fluka Chemie, Buchs, Switzerland) -stained left ventricular sections under polarized light. Areas of connective tissue network and myocytes were quantified by a semi-automated computer-based analysis system as described by Brooks et al. (1997). The results are presented as a percentage of collagen positive area per total myocardial area.

The expression of connective tissue growth factor in the heart was determined by methods described earlier (Finckenberg et al., 2003). Primary antibodies against connective tissue growth factor (anti-mouse connective tissue growth factor; Abcam, Cambridge, UK) were used. The intensity of connective tissue growth factor expression was evaluated by scoring the samples from 0 to 4 according to the amount of antibody positive label.

### 2.4. Biochemical determinations

Blood glucose was determined at the end of the experiment with Glucocard II Super test meter (KDK Corporation, Kyoto, Japan) and serum insulin by radioimmunoassay (Lino Research Inc, St Charles, MO, USA). Plasma aldosterone concentration was measured by radioimmunoassay according to the instructions of the manufacturer (DPC, Los Angeles, CA, USA). Natriuretic peptides from cardiac tissue samples were determined by radioimmunoassays and quantitative reverse transcription polymerase chain reaction (RT-PCR) as described below.

#### 2.4.1. Radioimmunoassays

The cardiac tissue samples (75–100 mg) were homogenized in 0.75 ml of the guanidine isothiocyanate extraction solution of the QuickPrep Total RNA kit (Amersham Biosciences). After centrifugation, 0.3 ml of the supernatant was subjected to SepPak $C_{18}$ extraction and radioimmunoassays and the remaining was used to obtain RNA for quantitative RT–PCR (see below).

Rat N-terminal fragment of ANP (NT-proANP) was determined by radioimmunoassay as described previously (Vuolteenaho et al., 1992). The sensitivity of the NT-proANP assay was 0.75 fmol/tube.

Rat BNP was assayed using a goat antiserum raised against a thyroglobulin conjugate of rat $BNP_{22–42}$ ($proBNP_{72–92}$) and horseshoe crab hemocyanin. Rat ANP, CNP, ET-1 or adrenomedullin were not recognized by the antiserum (cross-reaction < 0.001%). Synthetic $Tyr_0$-rat $proBNP_{72–92}$ was radioiodinated using chloramines-T and desalted by Sephadex G-25 gel filtration (Amersham Biosciencies). Final purification of the tracer was made by reverse-phase high performance liquid chromatography (HPLC) using a Symmetry $C_8$column (Waters) and a linear 30 min gradient from 10–40% acetonitrile in aqueous 0.1% trifluoroacetic acid. Synthetic rat BNP-45 was used as standard. The sensitivity of the BNP assay was 0.5 fmol/tube and the within and between assay coefficients of variation were <10% and 15%, respectively.

#### 2.4.2. Quantitative RT-PCR

Total RNA was extracted from rat cardiac tissue using QuickPrep Total RNA reagents from Amersham Biosciences. The cDNA first strand was synthesized with M-MuLV reverse transcriptase. The quantitative PCR reactions were performed with an ABI 7700 Sequence Detection System (Applied Biosystems) using the TaqMan® chemistry. The sense and antisense primers and the amplicons quantified corresponded to rat ANP mRNA (X00665) nucleotides 398–419, 467–450 and 421–439. The results were normalized to 18S RNA quantified from the same samples as described previously (Majalahti-Palviainen et al., 2000). The sequences of the sense and antisense primers, and the fluorogenic probes are listed in Table 1.

Table 1
Oligonucletides used in the real time quantitative PCR reactions

| Target | Sense primer | Antisense primer | Fluorogenic probe |
|---|---|---|---|
| Rat ANP | GAAAAGCAAACTGAGGGCTCTG | CCTACCCCCGAAGCAGCT | TCGCTGGCCCTCGGAGCCT |
| 18S | TGGTTGCAAAGCTGAAACTTAAAG | AGTCAAATTAAGCCGCAGGC | CCTGGTGGTGCCCTTCCGTCA |

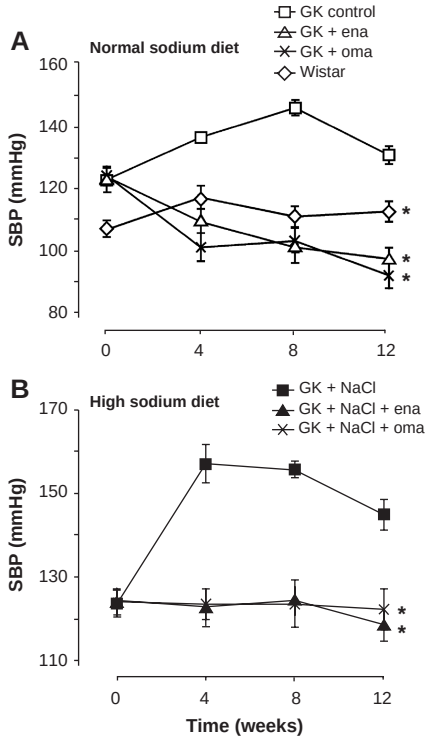


Fig. 1. The development of systolic blood pressure during normal-(A) and high-sodium diet (B). The systolic blood pressure was significantly increased in the untreated Goto–Kakizaki (GK) groups compared to the nondiabetic Wistar rats, and was further significantly elevated by the high-sodium diet. The blood pressure-lowering effects of enalapril- and omapatrilat-treatment were equal both during normal- and high-sodium diet. However, the high dietary salt impaired the antihypertensive effect of both drugs. *=$P<0.05$ vs. respective GK control group. SBP=systolic blood pressure, ena=enalapril, oma=omapatrilat.

### 2.5. Statistical analyses

Data were analyzed using analysis of variance (ANOVA) followed by Newman–Keul's post hoc test. When the data consisted of repeated measures at successive time-points (such as systolic blood pressure measurements) ANOVA for repeated measures and Student–Newman–Keul's post hoc analyses were used. A difference of $P<0.05$ was considered statistically significant. Results are presented as mean±S.E.M. unless otherwise stated.

Table 2B
Body weight, blood glucose, serum insulin, plasma aldosterone, cardiac connective growth factor score and collagen volume fraction in diabetic GK rats on a high-sodium diet

| | GK control ($n$=9) | GK+ena ($n$=8) | GK+oma ($n$=9) |
|---|---|---|---|
| Body weight (g) | 401±10 | 393±8 | 395±9 |
| Blood glucose (mmol/l) | 15.9±1.8 | 16.8±1.2 | 13.6±1.3 |
| Serum insulin (ng/ml) | 2.1±0.2 | 2.1±0.2 | 2.8±0.3 |
| Plasma aldosterone (μg/l) | 77.9±3.8 | 79.1±5.7 | 77.5±4.9 |
| Cardiac CTGF score (from 0 to 4) | 2.2±0.2 | 1.8±0.3 | 1.9±0.4 |
| Collagen volume fraction (%) | 3.0±0.3 | 3.0±0.4 | 3.1±0.4 |

Values are given as mean±S.E.M. CTGF = connective tissue growth factor.

## 3. Results

### 3.1. Systolic blood pressure

In the beginning of the experiment the systolic blood pressure averaged 124±3.4 mmHg in the GK groups, while it was 107±2.7 mmHg in the Wistar control group ($P<0.01$). During the experiment, the systolic blood pressure in the GK control group on a normal-sodium diet increased significantly compared to the nondiabetic Wistar rats (Fig. 1A). The high-sodium diet further increased the blood pressure (GK controls on a normal- vs. on a high-sodium diet, $P<0.05$).

There was no difference in the antihypertensive effect between enalapril and omapatrilat (Fig. 1A+B). Both drug treatments decreased the systolic blood pressure significantly as compared to the GK control rats during normal- as well as during high-sodium diet. However, as in the untreated GK group, the blood pressure level was increased by the high sodium intake also in the drug-treated groups (enalapril during normal- vs. high-sodium diet, $P<0.01$, and omapatrilat during normal- vs. high-sodium diet, $P<0.001$).

Table 2A
Body weight, blood glucose, serum insulin, plasma aldosterone, cardiac connective tissue growth factor score and collagen volume fraction in diabetic GK rats and control Wistar rats on a normal-sodium diet

| | GK+control ($n$=9) | GK+ena ($n$=8) | GK+oma ($n$=8) | Wistar ($n$=10) |
|---|---|---|---|---|
| Body weight (g) | 385±8 | 401±9 | 384±5 | 437±14 [b] |
| Blood glucose (mmol/l) | 17.9±2.4 | 13.5±1.9 | 13.7±2.7 | 7.5±0.5 [b] |
| Serum insulin (ng/ml) | 2.6±0.5 | 2.7±0.3 | 2.6±0.4 | 4.1±0.5 |
| Plasma aldosterone (μg/l) | 222.0±40.1 | 148.8±13.6 | 108.3±16.5 [a] | 179.9±19.3 |
| Cardiac CTGF score (from 0 to 4) | 2.1±0.4 | 1.0±0.3 [a] | 1.1±0.4 [a] | 0.2±0.1 [c] |
| Collagen volume fraction (%) | 3.0±0.4 | 2.8±0.5 | 2.7±0.3 | 2.5±0.2 |

Values are given as mean±S.E.M. [a] $P<0.05$, [b] $P<0.01$, [c] $P<0.001$ vs. GK control group. CTGF = connective tissue growth factor.

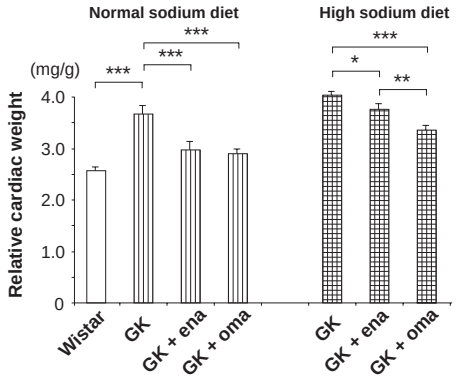


Fig. 2. The relative cardiac weight was significantly lower in the Wistar group than in the untreated GK groups. Both enalapril and omapatrilat equally prevented the development of cardiac hypertrophy as assessed by the relative cardiac weight during normal-sodium diet. During high-sodium diet omapatrilat reduced the relative cardiac weight more efficiently compared to enalapril treatment. *=$P<0.05$, **=$P<0.01$ and *** $P<0.001$. Ena=enalapril, oma=omapatrilat.

### 3.2. Body weight and heart weight

There were no significant differences in body weights between the GK groups regardless of dietary salt content or drug treatment. The Wistar rats, however, gained more weight during the study period in comparison with any of the GK groups (Tables 2A and B).

At the end of the experiment the Wistar rats had significantly lower relative cardiac weight (heart weight to body weight ratio) in comparison with the GK control rats (Fig. 2). The high-sodium diet tended to induce cardiac hypertrophy as assessed by relative cardiac weight, but the difference between the GK control groups on a normal- or a high-sodium diet did not reach statistical significance (3.7±0.16 and 4.0±0.089, respectively, $P=0.06$).

During normal-sodium diet, enalapril and omapatrilat equally prevented the development of cardiac hypertrophy in GK rats, and during high-sodium diet both enalapril and omapatrilat significantly lowered the relative cardiac weight as compared to the controls. However, omapatrilat was more effective in reducing the relative cardiac weight than enalapril ($P<0.01$).

Table 3A
ECHO-data in diabetic GK rats and control Wistar rats on a normal-sodium diet

| | GK control ($n=9$) | GK+ena ($n=8$) | GK+oma ($n=8$) | Wistar ($n=10$) |
|---|---|---|---|---|
| Heart rate | 217±10 | 193±15 | 184±13 | 208±5 |
| IVS (mm) | 1.3±0.05 | 1.2±0.03 | 1.1±0.04 [b] | 1.2±0.02 [a] |
| PW (mm) | 1.4±0.05 | 1.4±0.04 | 1.4±0.04 | 1.3±0.02 |
| LVESD (mm) | 8.2±0.4 | 7.7±0.2 | 7.1±0.2 [a] | 5.8±0.1 [c] |
| LVEDD (mm) | 9.5±0.2 | 9.3±0.2 | 9.2±0.1 | 8.2±0.1 [c] |
| LVEDV (μl) | 713±87 | 703±37 | 581±43 | 550±44 |
| FS (%) | 30±4 | 35±3 | 34±4 | 47±2 [b] |
| E/A ratio | 4.4±0.8 | 3.6±0.6 | 4.9±0.5 | 2.4±0.3 [a] |
| E deceleration slope ($m/s^2$) | 22.2±1.9 | 17.6±2.2 | 20.6±1.5 | 16.0±1.4 |

Values are given as mean±S.E.M. IVS=intraventricular septum, PW=posterior wall, LVESD=left ventricular end systolic diameter, LVEDD=left ventricular end diastolic diameter, LVEDV=left ventricular end diastolic volume, FS=fractional shortening, E/A ratio=peak transmitral (E wave) flow/A wave flow. [a] $P<0.05$, [b] $P<0.01$ and [c] $P<0.001$ vs. GK control group.

Table 3B
ECHO-data recorded in diabetic GK rats on a high-sodium diet

| | GK control ($n=9$) | GK+ena ($n=8$) | GK+oma ($n=9$) |
|---|---|---|---|
| Heart rate | 218±15 | 185±10 | 180±8 |
| IVS (mm) | 1.3±0.06 | 1.3±0.04 | 1.3±0.05 |
| PW (mm) | 1.4±0.08 | 1.4±0.04 | 1.4±0.06 |
| LVESD (mm) | 7.9±0.5 | 8.5±0.3 | 8.0±0.3 |
| LVEDD (mm) | 9.6±0.3 | 10.2±0.07 | 9.8±0.2 |
| LVEDV (μl) | 790±109 | 814±43 | 777±59 |
| LV mass (g) | 1.08±0.11 | 1.13±0.04 | 1.11±0.09 |
| FS (%) | 32±3 | 32±3 | 33±3 |

Values are given as mean±S.E.M. IVS=intraventricular septum, PW=posterior wall, LVESD=left ventricular end systolic diameter, LVEDD=left ventricular end diastolic diameter, LVEDV=left ventricular end diastolic volume, LV mass=left ventricular mass, FS=fractional shortening.

### 3.3. Echocardiography

Cardiac dimensions and performance parameters measured by transthoracic echocardiography are given in Tables 3A and B and Figs. 3 and 4.

The intraventricular septum thickness was increased in the GK control group compared to the nondiabetic Wistar group during normal-sodium diet (Table 3A). Furthermore, the GK rats had significantly larger left ventricular end systolic and diastolic diameters (Tables 3A and B), as well as echocardiographically measured left ventricular mass (Fig. 3 and Table 3B) as compared to the Wistar rats during both normal- and high-sodium diet ($P<0.001$, for each GK control group vs. Wistar), but the difference between normal- and high-sodium diet was not significant. Treatment with omapatrilat prevented the increase of the intraventricular septum thickness, left ventricular end systolic

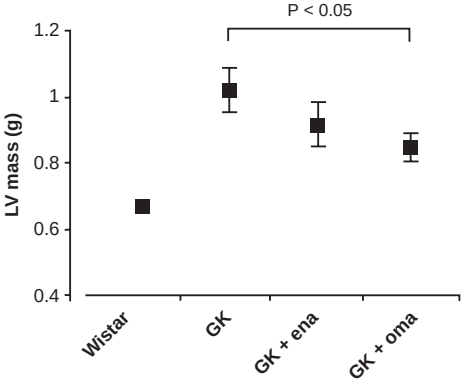


Fig. 3. The diabetic GK rats had significantly larger echocardiographically measured left ventricular mass than the Wistar control rats. During normal-sodium diet treatment with omapatrilat significantly prevented the increase in left ventricular mass in GK rats. LV mass=left ventricular mass, ena=enalapril, oma=omapatrilat.

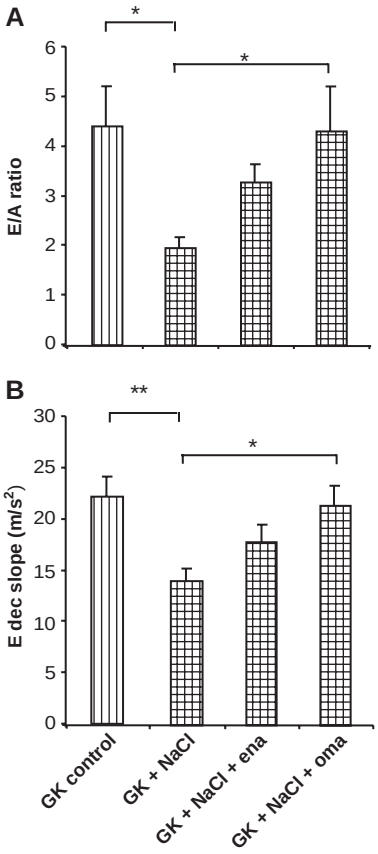


Fig. 4. The E/A-ratio (peak transmitral (E wave) flow to A wave flow) (A) and E wave deceleration slope (B) were measured by transthoracic echocardiography and used for assessment of cardiac diastolic function. The E/A ratio as well as the E wave deceleration slope were significantly reduced in the GK control group on a high-sodium diet compared to the untreated GK rats on a normal-sodium diet, indicating a development of diastolic dysfunction induced by the high dietary salt. Omapatrilat increased the E/A-ratio and the E wave deceleration slope compared to the GK controls during high-sodium diet, suggesting an improvement of the diastolic function. Dec=deceleration, ena=enalapril, oma=omapatrilat.

diameter (Table 3A) and left ventricular mass (Fig. 3) during normal-sodium diet, whereas these effects were lost during high-sodium diet (Table 3B). Enalapril did not cause any statistically significant changes in these parameters. There were no significant differences in the diastolic volumes among the groups studied (Tables 3A and B).

In order to study cardiac systolic function, we measured left ventricular fractional shortening from the parasternal window (Tables 3A and B). The fractional shortening was significantly impaired in all GK groups compared to the Wistar group. Neither dietary salt content nor drug treatment had a significant effect on fractional shortening.

The E/A-ratio and E wave deceleration slope were used for assessment of diastolic function. During normal-sodium diet the GK control rats had significantly higher E/A-ratio in comparison with the Wistar rats (Table 3A). Suggesting a development of left ventricular diastolic dysfunction, the high dietary sodium significantly reduced the E/A-ratio and the E wave deceleration velocity (Fig. 4). Omapatrilat, but not enalapril, significantly increased the E/A-ratio and the E wave deceleration slope as compared to the GK controls during high-sodium diet.

### 3.4. Collagen volume fraction and myocardial connective tissue growth factor expression

The myocardial collagen volume fraction was least in the nondiabetic Wistar rats but there was no statistically significant difference when compared to the GK groups (Tables 2A and B).

The expression of cardiac connective tissue growth factor was upregulated in the GK control groups as compared to the Wistar controls (Tables 2A and B). During normal-sodium diet the connective tissue growth factor score was markedly higher in the untreated GK than in the Wistar group.

Treatment with enalapril or omapatrilat equally reduced cardiac connective tissue growth factor overexpression in the groups on a normal-sodium diet. The high-sodium diet did not further increase the connective tissue growth factor expression, but impaired the effects of both drug treatments.

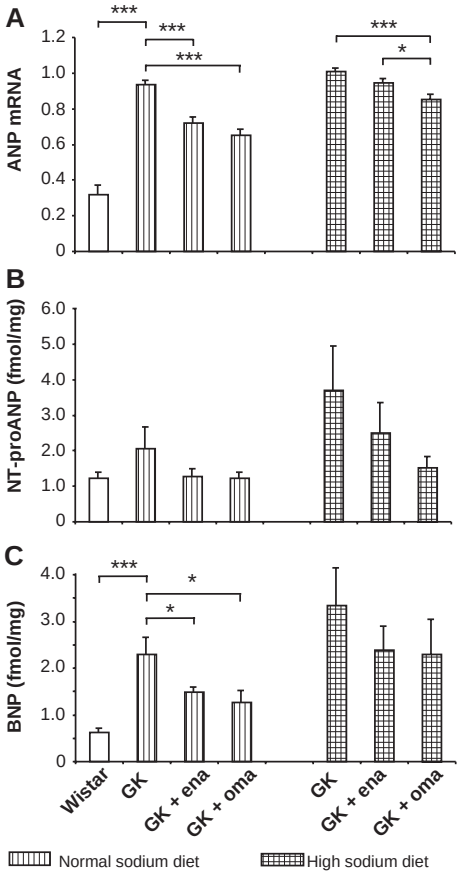


Fig. 5. The expression of both cardiac ANP mRNA (A) and BNP (C) were upregulated in the untreated GK rats compared to the nondiabetic Wistar rats. The high dietary sodium significantly increased the ANP mRNA expression in the heart (A) and also tended to elevate the expressions of cardiac NT-proANP (B) and BNP (C). During high-sodium diet the rats treated with omapatrilat had significantly reduced ANP mRNA expression compared both to the untreated and the enalapril-treated GK rats (A). *=$P<0.05$ and *** $P<0.001$. ANP=atrial natriuretic peptide, NT-proANP=N-terminal fragment of ANP, BNP=brain natriuretic peptide, ena=enalapril, oma=omapatrilat.

### 3.5. Cardiac natriuretic peptides

The cardiac ANP mRNA expression in the GK control group was 190% higher than in the Wistar group (Fig. 5). The high-sodium diet further increased the myocardial ANP mRNA expression ($P<0.05$, GK rats on a normal- vs. high-sodium diet). Treatment with enalapril or omapatrilat partially prevented the upregulation of ANP mRNA expression during normal-sodium diet. During high-sodium diet omapatrilat reduced the ANP mRNA expression as compared to the controls, and also as compared to the enalapril treated group. Enalapril had no effect on the ANP mRNA expression as compared to the GK controls during high-sodium diet.

The myocardial NT-proANP level was increased by 80% during the high dietary sodium. The drug treatments reduced the level of NT-pro-ANP, but the differences were not statistically significant.

The myocardial BNP level was increased by 270% in the GK rats on a normal- and 440% in the rats on a high-sodium diet, respectively, compared to the nondiabetic Wistars. The cardiac BNP tended to be increased by the high dietary sodium but the difference between the GK controls on a normal- or on a high-sodium diet did not reach statistical significance. Both drug treatments equally reduced the BNP levels as compared to the GK control animals during normal-sodium diet. During high-sodium diet the drug treatments did not have a significant effect on the myocardial BNP levels.

### 3.6. Blood glucose, serum insulin and plasma aldosterone

Blood glucose and serum insulin levels did not differ among the GK groups (Tables 2A and B). As expected, the blood glucose level was significantly higher in the GK groups as compared to the nondiabetic Wistars. There was no significant difference in serum insulin level between the GK and Wistar rats.

During the normal-sodium intake the plasma aldosterone level did not differ between the GK control group and the Wistar group (Tables 2A and B). Plasma aldosterone concentration was reduced by 65% in the GK control group on a high-sodium diet as compared to the normal-sodium diet GK controls ($P<0.01$). During normal-sodium diet omapatrilat reduced the plasma aldosterone level in comparison to the GK controls, but during high-sodium diet neither of the drugs influenced the level of aldosterone.

## 4. Discussion

The main findings of this study are:

1. The spontaneously diabetic GK rats developed hypertension and echocardiographic signs of cardiac diastolic dysfunction in response to high-sodium diet.
2. Both enalapril and omapatrilat normalized the systolic blood pressure during normal-sodium diet and decreased the blood pressure equally during high-sodium diet in GK rats.
3. Omapatrilat reduced cardiac hypertrophy as assessed by the relative cardiac weight and decreased volume overload as assessed by ANP mRNA more effectively than enalapril during high-sodium diet.
4. Omapatrilat reduced the echocardiographically measured left ventricular mass during normal-sodium diet and improved the diastolic function during high-sodium diet.

In addition to glucose metabolism abnormalities, GK rats develop several cardiovascular alterations typical of type 2 diabetes. Recently, endothelial dysfunction has been shown to participate in the pathogenesis of hypertension in GK rats (Cheng et al., 2001).

In our study, the GK rats developed higher systolic blood pressure than the nondiabetic Wistar control rats already in the absence of salt overload. As a surrogate marker for volume overload we measured cardiac ANP mRNA and BNP values which were increased in GK rats as compared to Wistar rats. Similar findings of increased ANP levels in GK rats have been reported by Vesely et al. (1999), who simultaneously with increased ANP levels found significantly larger blood volumes of GK rats compared to Wistar rats. Also in another diabetic rat model, the Zucker diabetic fatty rat, the expression of ANP mRNA was increased, probably due to hyperglycemia-induced volume expansion (Fredersdorf et al., 2004). Together with endothelial dysfunction and diabetic renal changes (Galli et al., 1999; Janssen et al., 1999; Riley et al., 1999; Schrijvers et al., 2004), hypervolemia makes GK rats prone to hypertension and development of cardiac hypertrophy.

The differences in echocardiographically measured mitral inflow (E/A ratio and E deceleration slope) in GK rats on a normal-sodium diet as compared to Wistar rats may, at least partly, reflect a hypervolemic state of the GK rats. In otherwise age-, and heart rate-matched animals hypervolemia and increased preload may cause a pseudo-normalization of the dynamic mitral inflow parameters, so that the E/A ratio and the E deceleration slope increases. The systolic function of the heart was impaired in GK rats compared to Wistar control rats as judged by a decrease in fractional shortening.

When high salt intake was combined to the pre-existing diabetes and hypertension, an exaggerated response was seen in some parameters evaluated: systolic blood pressure increased significantly compared to the GK rats on a normal-sodium diet, mitral inflow pattern changed towards diastolic dysfunction and cardiac ANP mRNA and BNP were increased further, although the difference in BNP did not reach statistical significance. The relative cardiac weight, connective tissue growth factor score or collagen volume fraction, in addition to echocardiographically measured intraventricular septum or posterior wall thickness, left ventricular mass or left ventricular diameters, on the other hand, did not show enhanced deterioration as a result of a high-sodium intake. As a response to salt- and volume-overload and hypertension, the plasma aldosterone levels were significantly lower during high-sodium diet compared to GK rats on a normal-sodium diet.

The high sodium content in the diet caused the mitral inflow velocity curves to shift towards pattern typical of diastolic dysfunction. In age-, weight-, and heart rate-matched GK rats, the E/A ratio diminished and the E wave deceleration slope decreased compared to GK rats on a normal-sodium diet. These changes indicate abnormal relaxation of the left ventricle associated with mildly increased left ventricular filling pressures even at rest.

Our aim in this study was to investigate whether the vasopeptidase inhibitor omapatrilat has additional cardioprotective effects in comparison with a pure angiotensin converting enzyme inhibitor, enalapril. During normal-sodium diet both medications were equally effective in normalizing the systolic blood pressure and in reducing the relative cardiac weight. However, only omapatrilat significantly decreased the intraventricular septum thickness, left ventricular end systolic diameter and left ventricular mass as compared to the GK control rats, suggesting that these effects of cardiac remodeling are not merely mediated by the lowering of blood pressure. Omapatrilat, but not enalapril, significantly reduced plasma aldosterone in these rats. Both drugs also reduced, but not abolished, the increased connective tissue growth factor expression seen in GK rats as compared to Wistar control rats. The insignificant differences in collagen volume fraction between the groups could relate to a too short study period to detect slow long-term structural changes in cardiac fibrosis, as even the difference between Wistar controls and untreated GK rats was not significant. The cardiac expression of both ANP mRNA and BNP were significantly lowered by enalapril and omapatrilat in accordance with the reduced afterload of the heart brought about by the decreased systolic blood pressure.

During normal-sodium diet there were no significant changes in mitral inflow pattern due to either enalapril- or omapatrilat-treatment. The high E/A ratio and increased E wave deceleration slope, most likely reflecting hypervolemia, persisted despite drug treatment as neither of the drugs has a significant diuretic effect. Cardiac systolic function was not altered by either medication, although there was a minor trend towards improvement during both enalapril- and omapatrilat-treatment.

During high-sodium diet both medications decreased, though not normalized, the systolic blood pressure to the same extent. Again, in line with the lowered systolic blood pressure, the ANP mRNA and BNP levels were decreased in medicated GK rats compared to untreated animals also during high-sodium intake, more notably by omapatrilat than by enalapril.

The antihypertrophic effect of the drugs was impaired by the high-sodium diet, but both drugs still reduced the relative cardiac weight compared to the untreated GK rats. However, the effect of omapatrilat was significantly greater than that of enalapril as evaluated by the difference in relative cardiac weight — despite the equal blood pressure-lowering effect. It has been suggested that inhibition of neutral endopeptidase may reduce hypertrophy by increasing autocrine and paracrine actions of natriuretic peptides (Bäcklund et al., 2003), and a selective neutral endopeptidase inhibitor has been demonstrated to reduce left ventricular hypertrophy without effects on systolic blood pressure in spontaneously hypertensive rats (Monopoli et al., 1991). In this study, the echocardiographically determined left ventricular mass and wall thickness were not affected by either medication during high salt intake and neither were the left ventricular diameters diminished—a discrepancy which may be explained by methodological differences; the relative cardiac weight takes into account the weight of the whole heart and the body weight of the animal, whereas echocardiography measures only selected parameters. Also, during high-sodium diet, the changes caused by medication may be too subtle or so slowly appearing that echocardiography does not yet detect them at this time point.

Furthermore, during high-sodium diet treatment with enalapril tended to ameliorate both the E/A ratio and the E-wave deceleration slope while omapatrilat reversed them to the level seen in GK rats on a normal-sodium diet. This finding suggests an improvement of the diastolic function by omapatrilat during high-sodium diet. The systolic function was not improved by either of the drugs during this study period.

The high-sodium diet impaired also the positive effect of enalapril and omapatrilat on the connective tissue growth factor score and collagen volume fraction, thus amplifying the adverse effect of high salt intake on the heart. Likewise, neither enalapril nor omapatrilat could further lower plasma aldosterone levels.

At the end of the experiment the rats were 20 weeks old, i.e., still young animals, in which the naturally occurring deterioration of the cardiovascular system was not readily detectable without an added insult of salt overload. Using isolated Langendorff-perfused hearts, El-Omar et al. (2004) demonstrated that no significant functional changes can be found in GK rat hearts under normoxic conditions while after a brief exposure to hypoxia a marked contractile defect could be seen both in the systolic and diastolic left ventricular function. Based on their experiments with insulin, they suggested that the dysfunction was primarily of metabolic origin (El-Omar et al., 2004). The GK rats have previously been shown to have an impaired glucose-stimulated insulin secretion (Mosén et al., 2005; Tourrel et al., 2002; Metz et al., 1999). The development of hyperinsulinemia in the GK rat is age-dependent (Witte et al., 2002). In our study hyperinsulinemia was not found in the GK rats despite hyperglycemia. In our experimental setting the diastolic dysfunction, caused by salt overloading and by associated changes in systolic blood pressure and volume load, could be corrected by medication, which interfered with both the renin-angiotensin and the neutral endopeptidase system. This suggests, that the direct metabolic changes caused by

the diabetic hyperglycemia are at least not the only targets in the treatment of diabetic cardiomyopathy.

It has been conclusively proved in several studies, that intensive blood pressure-treatment is beneficial in preventing the development of cardiovascular complications in diabetics (UKPDS, 1998; Hansson et al., 1998; Zanchetti and Ruilope, 2002; Ball, 2003). In our study, the lowering of systolic blood pressure alone did not explain the beneficial cardiac effects in vasopeptidase inhibitor-treated rats, as the blood pressure values attained with both enalapril and omapatrilat were equal. The results suggest that vasopeptidase inhibitors have cardioprotective potential beyond angiotensin converting enzyme inhibition and blood pressure-lowering properties. As a consequence of the complex pharmacology of the vasopeptidase inhibitors the exact cellular and molecular mechanisms through which these beneficial effects and, on the other hand, side effects are operating remain to be examined in future studies.

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