Creatine kinase and blood pressure: Clinical and therapeutic implications
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Creatine kinase inhibition with beta-guanidinopropionic acid reduces blood pressure in the spontaneously hypertensive rat

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Submitted
Chapter 9A

**ABSTRACT**

**Background** We have reported that creatine kinase is the main predictor of hypertension in the population and has a strong contribution to vascular contractility. Catalyzing the reaction: \( \text{MgADP} + \text{PCreatine} + \text{H}^+ \leftrightarrow \text{MgATP} + \text{Creatine} \), the enzyme provides ATP for sodium retention and vascular contractility. In this study, we hypothesized that inhibition of creatine kinase with beta-guanidinopropionic acid reduces blood pressure in the spontaneously hypertensive rat.

**Method** Male, 16-weeks-old spontaneously hypertensive rats (N=16) were randomly assigned to a standard diet with or without beta-guanidinopropionic acid. Blood pressure was measured weekly by the non-invasive tail-cuff method. After 4 weeks we assessed the effect on vasodilatory responses of mesenteric arteries in a wire myograph.

**Results** Treatment with beta-guanidinopropionic acid significantly reduced systolic and diastolic blood pressure compared to controls, by 42.7 (SD 5.5) and 35.3 (4.8) mm Hg respectively \((p<0.001)\). Beta-guanidinopropionic acid enhanced the vasodilatory response of rat mesenteric arteries to dinitrofluorobenzene, by 82.2\% \((p=0.008)\) a creatine kinase inhibitor. Moreover, incubation of isolated rat mesenteric arteries with 150 mg beta-guanidino-propionic acid induced a 25.7(4.4)\% vasodilation, demonstrating a decreased vascular contraction potential by creatine kinase inhibition.

**Conclusion** To our knowledge, we are the first to show that creatine kinase inhibition with beta-guanidinopropionic acid reduces blood pressure. Modulation of the creatine kinase-system might be a novel promising target for the treatment of hypertension.
BACKGROUND

Creatine kinase (CK) was proposed to affect pressor responses through rapid supply of ATP for energy demanding processes such as sodium retention, cardiovascular contractility, and remodelling of arteries. In line with this, serum CK was reported to be the main predictor of blood pressure in the population, independent of age, sex, body mass index, and ethnicity.

By catalyzing the reversible conversion of creatine into phosphocreatine, CK builds up large cellular reserves of phosphocreatine for temporal and spatial buffering of ATP, via the reaction: Creatine + MgATP ↔ Phosphocreatine + MgADP. ATP, generated by glycolysis and oxidative phosphorylation, is transported as phosphocreatine to cytosolic ATP-utilizing enzymes, including myosin light chain kinase and myosin-ATPase at contractile proteins and Ca$^{2+}$-ATPase and Na$^+$/$K^+$-ATPase at cellular membranes. Here, ATP is regenerated by cytosolic CK and used for vascular contractility and sodium retention.

The flux through the CK reaction can be inhibited by beta-guanidinopropionic acid (βGPA), a naturally occurring creatine analogue that reduces the flux by reducing cellular creatine uptake (Figure 1). βGPA is phosphorylated in cytoplasm, but both βGPA and phosphorylated βGPA are inefficient substrates for the CK reaction: in vitro $V_{\text{MAX}}$ values are <1% of the $V_{\text{MAX}}$ values of creatine and phosphocreatine. Therefore, we hypothesized that βGPA reduces blood pressure in the spontaneously hypertensive rat (SHR), an animal model for essential hypertension.

METHODS

An expanded description of the Methods section is available in the Data Supplement.

Animals

Fourteen-week-old male SHR (N=16, mean body weight 316.9 g) were obtained from Charles River (Maastricht, the Netherlands) and handled according to a protocol approved by the Animal Ethical Committee of the University of Amsterdam, the Netherlands, in conformity with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The SHR were divided in 2 groups. Group 1 (N=8) received pure chow (pellets). Group 2 (N=8) received identical chow containing βGPA (3%) (Pure bulk Vitamins and Dietary Supplements, Roseburg, Oregon, USA) during 4 weeks. Based
on pilot studies in our laboratory showing limited food intake with βGPA during the first
days of the experiment, daily food supply of controls was restricted during the first 10
days. Both groups received 15 grams of chow per day. Thereafter, food was supplied ad
libitum. Food intake and weight were measured daily.

Figure 1. Mechanism of action of beta-guanidinopropionic acid.
Beta-guanidinopropionic acid (βGPA) enters the cell instead of creatine through competitive inhibition of the creatine transporter. βGPA is phosphorylated by cytosolic creatine kinase (CK), but is an ineffective substrate, leading to reduced flux through the CK reaction. βGPA cannot be phosphorylated by mitochondrial CK. Cr, creatine; CrP, phosphocreatine; CM, cellular membrane; MEM, mitochondrial outer membrane; MIM, mitochondrial inner membrane.

Tail-cuff blood pressure measurements
Standardized tail-cuff blood pressure measurements of conscious SHR were performed once weekly with the CODA™ system (Kent Scientific Corporation, Torrington, CT, USA) after a handling period of 2 weeks. The animals were restrained in a transparent animal holder and placed on a heating pad. Body temperature was measured regularly
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to maintain a temperature of 35 to 37ºC. The rat was left untouched and fixated for 5 minutes before placing the tail-cuffs. Then, the tail-cuffs were placed over the tail slightly below the tail base. Based on results from our pilot studies, in which we estimated the number of measurements after which the standard deviation remained constant (data not shown), we performed 10 repeated measurement cycles per rat per week. The mean of measurement cycles 6 to 10 was used for further analysis. During the measurements, care was taken to ensure minimal stress for the animals. The animals were held in the holder for a maximum of 20 minutes.

**Anesthesia, blood draw, and tissue harvesting**
At baseline and after 4 weeks blood was drawn from the tail vein of each rat for estimation of CK, creatinine, sodium, potassium, urea, glucose, total cholesterol, HDL, and triglycerides. After 4 weeks of intervention the animals were anesthetised with ketamine (90 mg/kg)–dexmedetomidine (0.125 mg/kg)–atropine (0.05 mg/kg) (KMA) through intraperitoneal injection for tissue harvesting. Mesenteric arteries (3rd order) were prepared for tension recording with a four-channel wire myograph (Danisch Myo Technology, Copenhagen, Denmark). In addition, aorta segments were mounted in an organ bath and force readout was recorded via a PowerLab data acquisition system (AD Instruments, Castle Hill, Australia).

**Data analysis and statistics**
The primary outcome was the difference in blood pressure of SHRs after intervention with βGPA (3%) versus untreated SHRs. Based on treatment of adult SHRs with other antihypertensive drugs we expected a decrease of 16 mm Hg (SD 7) after 4 weeks of intervention,\(^{10}\) and calculated we needed 6 rats in each group with a 2-tailed \(\alpha=0.05\) and \(1-\beta=0.80\). Secondary outcomes were differences in vasodilatory responses of isolated mesenteric arteries in a wire myograph. For the myograph experiments, based on an expected mean SD of 0.6 with a biologically relevant decrease of 1 mN in \(E_{MAX}\), we calculated 8 rats would be needed. Thus, we included 8 rats in each group, 16 rats in total. Data are presented as mean ± SEM with N being the number of individual rats. Peak contraction values during the myography experiments were determined and expressed as relative tension (mN*mm\(^{-1}\)). Comparisons between groups were performed using the Student’s \(t\)-test. When the data consisted of repeated observations in time, one-way Repeated Measures ANOVA was used to investigate between-group differences. Statistical analyses of myograph experiments were performed using Prism
(Graphpad Prism Software, San Diego, CA, USA). Other analyses were performed using SPSS Statistical Software, version 18.0.

**RESULTS**

**Body weight, food intake, and general appearance**
All animals appeared healthy and displayed normally physical activity. Food intake was reduced during the first four days with βGPA (3%) compared to controls, despite the food restriction as described in the methods section. After four days food intake was similar in both groups (data not shown). Treatment with βGPA (3%) reduced body weight during the first week. After one week body weight increased with a similar rate in both groups: βGPA 338.3 (5.6) versus control 355.6 (8.8) g (p=0.11) (Figure 2, panel A).

![Figure 2. Body weight, systolic and diastolic blood pressure.](image)

Line graphs showing body weight (A), systolic (B), and diastolic blood pressure (C) of spontaneously hypertensive rats from 16 to 20 weeks of age, i.e. from baseline to 4 weeks of intervention with βGPA (3%) (N=8) (●) or control chow (N=8) (■). Data are presented as mean values with standard error bars.
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Systolic and diastolic blood pressure, and heart rate
At baseline systolic and diastolic blood pressure were equal in both groups. After 4 weeks systolic as well as diastolic blood pressure of SHRs treated with βGPA were decreased compared to control SHRs by 42.7 (5.5) (p<0.001) and 39/2 (4.1) mm Hg (p=0.004) respectively (Figure 2, panel B and C). Heart rate was similar in both groups at all time points (data not shown).

Heart weight and heart weight/ body weight ratio
Heart weight (βGPA 987.5 (93.4) vs control 937.5 (101.6) mg, p=0.72) and heart weight/ body weight ratio (βGPA 2.9 (0.3) vs control 2.7 (0.3), p=0.65) were not significantly different between groups.

Mesenteric arteries and aorta: vessel diameter, contractility, and vasodilatory responses
The diameter of βGPA treated vessels was increased compared to control vessels; 299.0 (9.1) vs 274.8 (5.5) µm respectively (p=0.039). Maximum contractility of isolated mesenteric resistance arteries, induced by norepinephrine (10 µM), was not significantly different between controls and treated animals, 3.9 (0.4) and 4.5 (0.2) mN*mm⁻¹ (p=0.201). After precontraction with KPSS and norepinephrine (10 µM), there were no significant differences between concentration-response curves of mesenteric arteries in response to methacholine (p=0.12) and sodium nitroprusside (p=0.33) between groups, although there was a trend for increased vasodilation by methacholine after βGPA (Figure 3, panel A). The vasodilatory response to the CK-inhibitor DNFB was enhanced by 82.2% after treatment with βGPA (Figure 3, panel B). Incubation with 150 mg βGPA induced a 25.7 (4.4)% vasodilation (p=0.008) in both groups (Figure 3, panel C). In the aorta the vasodilatory response to methacholine was increased by 27.6% (p=0.007), which was partially augmented by L-NNA (p=0.24) (Figure 4).

Biochemical analyses
Plasma levels at baseline and after 4 weeks of intervention with βGPA, including serum CK, sodium, potassium, urea, non fasting glucose, and triglycerides were not significantly different between groups (Table 1). Non fasting cholesterol and HDL were increased by βGPA. Plasma creatinine concentration was significantly reduced by 49.1% after βGPA. This finding shows that inhibition of cellular creatine uptake by βGPA is effective, as
intracellular creatine is nonezymatically converted to creatinine, which diffuses out of the cells and is excreted by the kidneys.\textsuperscript{11}

Myocardial ATP concentration was reduced after 4 weeks of βGPA compared to controls; 1.6 (0.1) vs. 1.9 (0.1) pmol per μg protein ($p=0.05$). The ATP/ADP ratio was similar in both groups: 3.33 (0.4) and 3.42 (0.2) in treated and control SHRs respectively. As expected, the ATP content of skeletal muscle was significantly reduced after βGPA; 1.05 (0.04) vs 1.35 (0.07) pmol per mg protein ($p<0.001$).

As expected given the blood pressure decrease, relative renin mRNA in the kidney cortex was 1.6 fold increased after βGPA compared to controls 1.7 (0.2) vs. 1.1 (0.2) ($p=0.07$).

**Figure 3.** Cumulative concentration-response curves in mesenteric arteries of spontaneously hypertensive rats.
Relaxation induced by cumulative concentrations of DNFB (A), βGPA (B), methacholine (C), and sodium nitroprusside (SNP) (D) in isolated mesenteric arteries of SHR treated with βGPA (3%) (●) and control SHR (■). Data are presented as mean values with standard error bars, N=5-8.
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Figure 4. Cumulative concentration-response curves in aortas of spontaneously hypertensive rats.
Relaxation induced by cumulative concentrations of methacholine, without incubation with LNNA (A) and after incubation with LNNA (10 μM) (B), in isolated aortas of SHR treated with βGPA (3%) (■) and control SHR (●). Data are presented as mean values with standard error bars, N=7.

Table 1. Plasma biochemical parameters.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Intervention</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>βGPA</td>
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<tr>
<td>Creatine kinase (IU/L)</td>
<td>113.5 (53.4)</td>
<td>109.4 (40.2)</td>
</tr>
<tr>
<td>Creatinine (μmol/L)</td>
<td>22.0 (2.7)</td>
<td>22.8 (2.8)</td>
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<tr>
<td>Urea (mmol/L)</td>
<td>6.5 (1.0)</td>
<td>6.9 (1.1)</td>
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<tr>
<td>Sodium (mmol/L)</td>
<td>143.9 (1.1)</td>
<td>143.2 (1.5)</td>
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<tr>
<td>Glucose (mmol/L) ‡</td>
<td>9.8 (1.2)</td>
<td>9.4 (1.0)</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L) ‡</td>
<td>1.6 (0.15)</td>
<td>1.6 (0.10)</td>
</tr>
<tr>
<td>HDL (mmol/L) ‡</td>
<td>1.3 (0.11)</td>
<td>1.3 (0.56)</td>
</tr>
<tr>
<td>Triglycerides (mmol/L) ‡</td>
<td>0.78 (0.25)</td>
<td>0.87 (0.19)</td>
</tr>
</tbody>
</table>

Plasma biochemical parameters of treated and control spontaneously hypertensive rats before and after 4 weeks of intervention with βGPA (3%). Data are means with standard deviation in square brackets. * P<0.01 vs all other groups † Intracellular creatine is nonenzymatically converted to creatinine, which diffuses into plasma. ‡ βGPA inhibits cellular creatine uptake, leading to decreased formation of creatinine and decreased plasma creatinine levels. ‡ non fasting
DISCUSSION

In the present study we show that βGPA, an inhibitor of the CK reaction flux, effectively reduced systolic and diastolic blood pressure of SHR by 42.7 (5.5) and 39.2 (4.1) mm Hg respectively compared to controls. To our knowledge, this is the first report on the blood pressure lowering effect of βGPA, an effective inhibitor of the flux through the CK reaction. In agreement, previous studies showed that serum CK was the main predictor of blood pressure in the population and that inhibition of intravascular CK in isolated human resistance arteries reduced vascular contractility.2,6,12 Furthermore, high CK activity is found in the heart and the aorta of the SHR, prior to the development of hypertension.13-15

Several underlying mechanisms may be involved in the blood pressure lowering effect of βGPA in the SHR. These mechanisms all start with the inhibition of cellular creatine uptake, leading to a reduced flux through the CK reaction, with associated decreases of phosphocreatine, the main cellular ATP buffer, and ATP levels itself.8,9,16 The effective inhibition of cellular creatine uptake in this study is evident, as serum creatinine concentration was reduced after βGPA, showing reduced intracellular conversion from creatine.11

In vascular smooth muscle, CK probably functions as an energy transducer at the contractile proteins, myosin-ATPase and myosin light chain kinase, where it rapidly provides ATP for muscle contractions.1-3 The SHR is known to display greater vascular myosin-ATPase and myosin light chain kinase activity, with greater and faster shortening of arterial muscle, greater contractile responses to agonist stimulation, and reduced responses to relaxing agents compared to normotensive controls.17,18 Thus, inhibition of vascular CK by βGPA may decrease ATP supply to the contractile proteins, leading to reduced vascular contractility. In agreement with this, the mesenteric arteries of treated SHR in this study showed reduced residual contractility after the CK-inhibitor dinitrofluorobenzene, pointing to lower CK activity in these vessels. Moreover, in vitro acute incubation of mesenteric arteries with βGPA induced vasodilation in both groups. Even a small decrease in the contractility of vascular smooth muscle could have a profound effect on arterial pressure, as expressed in Poisseuille's formula, blood flow and resistance in vivo are markedly affected by small changes in vessel caliber.19

As a signaling molecule for vascular vasodilatory responses, the availability of nitric oxide (NO) may be enhanced by βGPA. High CK activity is thought to be associated with reduced NO biosynthesis, and NO-dependent functions, through reducing the
bioavailability of L-arginine. Creatine and NO are both synthesized from L-arginine, but creatine synthesis demands nearly 10 times the flux of plasma L-arginine compared to NO-synthesis.20 As cellular creatine uptake is inhibited by βGPA, there may be a relative creatine excess, leading to increased availability of arginine for nitric oxide synthesis.2,21 We studied NO-dependent relaxation in the mesenteric arteries via cumulative addition of methacholine on noradrenaline contraction in a high K⁺ depolarizing buffer. The mesenteric arteries displayed no significant influence of βGPA treatment on NO-induced relaxation. A possible explanation for the lack of effect of βGPA on NO-availability in the mesenteric arteries is the small measuring window for relaxation. By using a high K⁺ depolarizing buffer, the responses of endothelium-derived hyperpolarizing factor are abrogated, which include the main relaxation potential of mesenteric arteries, leaving mainly NO-dependent relaxation. In the aorta the NO-dependent vasodilatory response to methacholine was improved by βGPA. This response was almost completely blocked by LNNA, showing NO-dependency and pointing to increased NO availability after βGPA.

Furthermore, renal CK is thought be involved in sodium retention, as the enzyme provides ATP for sodium reabsorption at tubular basolateral Na⁺/K⁺-ATPases.1,22,23 As hypertension in the SHR is partly salt-sensitive,24 inhibition of renal CK by βGPA may decrease sodium reabsortion in the renal tubulus. Whether this contributed to the blood pressure decrease by βGPA remains to be determined.

Finally, it is reported that in striated skeletal muscle, inhibition of CK by βGPA induces a shift from type II to type I fiber predominance with increased oxidative metabolism, which is already found after 3 weeks of intervention with βGPA (1%) in the diet.25,26 Type I fiber predominance of skeletal muscle is associated with increased capillary density and lower peripheral resistance and lower blood pressures.27-29 In agreement with this, skeletal muscle type II fiber predominance is associated with hypertension.30,31 Moreover, evidence indicates a slow-to-fast fiber type transition prior to the development of hypertension in SHR compared to their normotensive counterparts.32 Thus, although beyond the scope of this study, evidence suggest that βGPA treatment may have altered skeletal muscle characteristics of the SHR leading to lower blood pressures.

All experimental animals appeared healthy and normally active. However, some studies reported myocardial hypertrophy after βGPA in normotensive rodents. We cannot exclude that βGPA may induce cardiomyopathy and heart failure, although heart weight and heart weight to body weight ratio were unchanged. Moreover, as the SHR is known to develop hypertensive cardiomyopathy between the age of 6 and 24
months, reducing blood pressure with βGPA may even prevent this complication of hypertension. As the SHR has high CK activity, we used a relatively high dose of βGPA of 3% in the diet compared to 1 to 2% in previous studies that investigated the effect of βGPA on skeletal muscle or heart function. We found a reduced myocardial ATP content with this relatively high dose. However, meta-analysis of previous studies showed that myocardial ATP content of normotensive rodents was not significantly reduced after lower doses of βGPA. Thus, a lower dose may be more selectively inhibiting vascular and kidney CK activity, as these activities are only 9 to 13% of myocardial CK. In dose finding experiments (unpublished data), we found that βGPA 1% reduced blood pressure of SHR.

This study has several strengths and limitations. One strength is that we standardized the tail cuff blood pressure measurements, according to previous pilot studies. The animals were all handled thoroughly during two weeks prior to the experiment and we took care that there was no difference in baseline blood pressure between both groups. All blood pressure measurements were performed under identical conditions. However, we cannot exclude stress from the restrainer. This is a limitation of the tail cuff method. Second, as pilot experiments showed weight loss with βGPA during the first experimental days, all animals were housed individually in order to assess individual food intake accurately.

Perspectives
It is well known that current antihypertensive agents are far from satisfactory, as more than 50% of patients does not achieve blood pressure control despite treatment. Especially certain population subgroups with relatively high tissue and serum CK activity, such as black people, suffer a greater burden of hypertension, with a higher prevalence of its complications, including stroke, heart failure, and hypertensive renal disease. Moreover, hypertension in blacks remains more difficult to treat. Therefore, new classes of antihypertensive drugs that act via new mechanisms are urgently needed. The results of this study show that inhibition of the CK-system with βGPA in SHR markedly reduces blood pressure. Together with previous reports on the association of CK with blood pressure, vascular contractility, and treatment failure, these results implicate that interference in the CK-system may be a novel promising target for antihypertensive treatment.
Creatine kinase inhibition reduces blood pressure

REFERENCES


Supplemental data to creatine kinase inhibition with beta-guanidinopropionic acid reduces blood pressure in the spontaneously hypertensive rat

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METHODS

Materials
Beta-guanidinopropionic acid was obtained from Purebulk Vitamins and Dietary Supplements (Roseburg, Oregon, USA). Before using βGPA in the experiments, purity was tested with NMR (VU, Amsterdam, Netherlands), showing a purity of > 99%. In addition, the substance was tested for the presence of cyanide compounds (Omegon Laboratory, Amsterdam), which were not present (<1.0 mg/kg). The cyano-group in cyanamide, one of the compounds used in the formation of βGPA, provides a possible source of cyanide. Methacholine (MCH), norepinephrine (NE), phenylephrine (PHE), sodium nitroprusside (SNP), amlodipine, dinitrofluorobenzene (DNFB), N\(^\text{G}\)-nitro-L-Arginine (L-NNA), and RNA\textit{later} were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

Blood sampling
At start of the experimental protocol and after 4 weeks of the diet, blood was drawn from the tail vein of each rat for estimation of CK, creatinine, sodium, potassium, urea, glucose, total cholesterol, HDL, and triglycerides. Prior to the first blood draw, rats were accustomed to laboratory handling procedures. Rats were anesthetized with isoflurane (3%) and oxygen (1 l/min), and placed on a warming pad. Blood was drawn from the tail vein, placed on ice immediately, and centrifuged at 4°C within one hour, according to the blood sampling method described for minimizing CK variability in rodents.\(^{11}\)

Anesthesia and tissue sampling
Animals were anesthetised with ketamine (90 mg/kg)–dexmedetomidine (0.125 mg/kg)–atropine (0.05 mg/kg) (KMA) through intraperitoneal injection. After exposing the abdominal and thoracic region of the rat, the heart, liver, kidney, musculus quadriceps femoris, and brain were rapidly excised, immediately rinsed in cold PBS buffer, snap frozen in liquid nitrogen, and stored in -80°C. The frozen heart and the lungs were weighted. The cortex of the right kidney was cut into pieces of 5 mm\(^3\) and the samples were incubated overnight in RNA\textit{later} at 4°C. The next day the RNA\textit{later} was removed and the samples were stored at 80°C.
**Resistance artery preparation and tension recording**

Mesenteric arteries were carefully excised and immediately placed into cold (4°C), oxygenated physiological salt solution (PSS) consisting of (in mmol/L) 118.2 NaCl, 24.8 NaHCO₃, 4.6 KCl, 1.2 KH₂PO₄, 1.2 MgSO₄, 2 CaCl₂, 0.26 EDTA, 50 HEPES, and 5.6 glucose. Vessels were dissected under a microscope and cleaned of adherent adipose and connective tissues. Three to four segments of 2 mm were mounted on 40-μm stainless steel wires. The segments were then transferred in to organ baths of a four-channel wire myograph (Danish Myo Technology, Copenhagen, Denmark). The myograph bath contained PSS at 37°C aerated with carbogen (5% CO₂/95% O₂). Then the vessels were subjected to a normalization procedure according to Mulvany and Halpern (1977).¹² The individual circumference was adjusted to 90% of the value that the particular vessel would have had at a transmural pressure of 100 mm Hg. Afterwards, the arteries were equilibrated for 20 minutes. The buffer was replaced every 10 minutes. Maximum contractility was induced in duplicate with norepinephrine (10 μM) in KCl (125 mM) – substituted PSS (KPSS). After washing and 20 minutes of rest, the vessels were precontracted with the α₁-adrenoreceptor agonist phenylephrine (10 μM). After a steady level of contraction force was attained, one concentration (10 μM) of the endothelium-dependent vasodilator methacholine was added to assess the endothelial integrity. After arteries had been washed and reequilibrated in PSS, cumulative concentration-response curves for methacholine (1 nM to 0.1 mM), sodium nitroprusside (1 nM to 0.1 mM), amlodipine (10 nM to 1 μM), and βGPA (25 mg to 150 mg) were performed. Finally, the irreversible CK inhibitor dinitrofluorobenzene (DNFB 1 μM to 1 mM) was added. Stock solutions of reagents (except DNFB) were prepared fresh daily in distilled water and diluted serially for use in myograph baths. DNFB was prepared as a 10-1 M stock solution in DMSO and further diluted daily in DMSO upon use in the myograph bath.

**Aorta preparation and tension recording**

The thoracic aorta was carefully excised and immediately placed in Krebs-Henseleit buffer (in mmol/L) 118.0 NaCl, 4.6 KCl, 25.0 NaHCO₃, 1.2 MgSO₄, 1.8 CaCl₂, 1.1 KH₂PO₄ and 5.6 glucose at room temperature, aerated with carbogen, pH 7.4. The aorta was cut into 2 mm segments, and mounted between two stainless steel hooks in an organ bath, containing 5 mL aerated Krebs buffer at 37°C. The segments were attached to a force transducer, and force readout was recorded via a PowerLab data acquisition system (AD Instruments, Castle Hill, Australia). The aorta segments were equilibrated for 1 hour at
an isotonic resting tension of 10 mN, which was maintained throughout the experiment. Then, the preparations were contracted twice for 10 min with a depolarizing high K⁺ Krebs-Henseleit solution (40 mmol/L NaCl was replaced by 40 mmol/L KCl) with intermediate washing steps of 20 min intervals. Subsequently, the vessels were precontracted with the α1-adrenoceptor agonist phenylephrine (1 μM). After reaching a steady level of >60% contraction compared with previous K⁺-induced depolarization contraction, the endothelium-dependent vasodilator methacholine (10 μM) was added to assess the endothelial integrity. After washing, again 40 mmol/L K⁺ was added to the vessel segments to obtain the maximal contractile response. After the aortas had been washed and reequilibrated during 30 minutes, a cumulative concentration-response curve methacholine (1 nM to 0.1 mM) were performed. To assess nitric oxide dependent vasodilation, one segment was incubated with the nitric oxide inhibitor L-NNA (10 μM) during 30 minutes prior to the addition of methacholine (1 nM to 0.1 mM).

Biochemical analysis

All plasma analyses were performed on a Modular Cobas 8000 (Roche Diagnostics, Darmstadt, Germany). Plasma levels of creatine kinase, glucose, total cholesterol and triglycerides were measured by enzymatic spectrofoctometric methods; high-density lipoprotein – cholesterol colorimetric/ spectrofotometric; plasma creatinine and urea with kinetic/spectofotometric methods; and sodium and potassium were estimated with Indirect Ion-Selective Electrode methods.

For the ATP determinations of heart and quadriceps muscle, tissue was grinded in liquid nitrogen, the powder transferred to 200 μl of 0.4 M perchloric acid (HClO₄), centrifuged at 10,000 x g at 4°C, and neutralized with 5 M potassium bicarbonate (K₂CO₃). The supernatant was removed from the pelleted cell fragments and immediately placed on ice for 10 minutes. After centrifugation at 10,000 x g at 4°C, the supernatant was assayed by HPLC. The pelleted cell fragments were stored with 0.2 M sodium hydroxide (NaOH) and used for protein determination by the BCA assay (Pierce).

For estimation of transcriptional activity for renin mRNA of the kidney cortex, total RNA was isolated from frozen-thawed rat kidney cortex, using Trizol reagent (GibcoBRL), and was quantified using micro-spectrophotometry (NanoDrop Technologies, Wilmington, USA). Thereafter, cDNA was synthesized from extracted RNA using QuantiTect Reverse Transcription Kit (USA, Qiagen), in which genomic DNA is removed by genomic DNA wipeout buffer before RT reaction. Quantitative real-time PCRs were conducted in a total volume of 20 μL and 10 ng of cDNA was used for each real-time
PCR reaction using a Step-One cycler Applied Biosystems (UK, Applied Biosystems), and the SYBR ® Green PCR Master Mix (UK, Applied Biosystems, catalogue no: 4385610) as per manufacturer's recommendations. The Intron-spanning oligonucleotide primers for qPCR were designed with NCBI (Primer-BLAST). The following cycling conditions were used [95ºC for 10 min, (95ºC for 15 s, 60ºC for 60 s) × 40 cycles]. β-actin, B2M and HPRT-1 DNA quantitation was performed in parallel on all samples in order to determine the actual input amount of cDNA and were used as endogenous references to normalize variations in DNA recovery and amplification efficiency.

A 2-fold dilution series was created from a random pool of cDNA from our sample groups. The PCR efficiency and correlation coefficients \( (R^2) \) of each primer pair were generated using the slopes of the standard curves. The efficiencies were calculated by the formula: efficiency (%) = \( 10^{(-1/\text{slope})} - 1 \) * 100. For a correct interpretation of the real-time PCR results, all data has been normalized which is achieved by calculating the geometric mean of the three stable reference genes.