Late Treatment With Ramipril Increases Survival in Old Spontaneously Hypertensive Rats

Wolfgang Linz, Paulus Wohlfart, Bernward A. Schoelkens, Reinhard H.A. Becker, Tadeusz Malinski, Gabriele Wiemer

Abstract—Spontaneously hypertensive rats (SHR) begin to die from cardiovascular complications at \( \approx 15 \) months of age. We tested whether chronic ACE-inhibitor treatment would extend the lifespan of such old animals. We also studied cardiac hypertrophy and function, endothelial function and expression, and activity of NO synthase (eNOS). One hundred 15-month-old SHR were randomized into 3 groups, control (n=10), placebo-treated (n=45), and ramipril-treated with an antihypertensive dose of 1 mg \( \cdot \) kg \(^{-1} \) \( \cdot \) d \(^{-1} \) in drinking water (n=45). Ex vivo experiments were performed after 15 months (control) and 21 months, when \( \approx 80\% \) of the placebo group had died. Late treatment with ramipril significantly extended lifespan of the animals from 21 to 30 months. Fully established cardiac hypertrophy, observed in placebo-treated animals and in controls, was significantly reversed by ramipril treatment. In isolated working hearts, a significantly improved function associated with increased cardiac eNOS expression was seen versus placebo and control hearts. Endothelial dysfunction in isolated aortic rings from control and placebo-treated SHR was significantly improved by ACE inhibition and associated with enhanced NO release. Late treatment of SHR with the ACE inhibitor ramipril extended lifespan from 21 to 30 months, which is comparable to the lifespan of untreated normotensive Wistar-Kyoto rats. This lifespan extension, probably due to blood pressure reduction, correlated with increased eNOS expression and activity followed by a regression of left ventricular hypertrophy and cardiac and vascular dysfunction. (Hypertension. 1999;34:291-295.)

Key Words: ramipril \( \cdot \) cardiac function \( \cdot \) hypertrophy \( \cdot \) endothelium \( \cdot \) rats, inbred SHR \( \cdot \) nitric-oxide synthase, endothelial

The spontaneously hypertensive rat (SHR) is an animal model of nature. It develops high blood pressure (BP) that has similarities to essential hypertension in humans. SHR die from cardiovascular complications at an age of about 15 months after a long period of stable, compensated cardiac and arterial hypertrophy as a result of persistent hypertension. The cardiovascular complications mainly involve ventricular fibrillations resulting from multiple microinfarctions and heart failure.

Some data obtained in genetically hypertensive rats point to a prolongation of survival through angiotensin-converting enzyme (ACE) inhibition. Short-term antihypertensive treatment of SHR between 6 and 10 weeks of age with the ACE inhibitor perindopril appeared to extend their lifespan. In an earlier study, male SHR were treated with captopril from the ages of 12, 18, or 21 months until 24 months. The degree to which captopril prevented myocardial dysfunction appeared to be related to the age at which captopril treatment was initiated and the duration of captopril administration.

Recently, we showed that lifelong ACE inhibition with ramipril doubles life expectancy in stroke-prone SHR, which matches the life expectancy of normotensive Wistar-Kyoto rats (WKY). This effect correlated with the prevention of the development of hypertension, of cardiac left ventricular hypertrophy most likely associated with improvement of myocardial function, and with the preservation of endothelial function. The latter was accompanied by enhanced upregulation of endothelial NO synthase (eNOS) expression and activity. Inspired by the outcome of this prevention study, the present study investigated whether late treatment of SHR with the ACE inhibitor ramipril is able to reverse fully developed cardiac hypertrophy and endothelial dysfunction, and, thus, improve survival.

Methods

Housing and Animal Care

Male SHR were purchased from Møllegaard, Denmark, and housed 3 per cage under standardized conditions of temperature, humidity, and light. The rats had free access to standard diet (Altromin Maintenance Diet 1320, sodium content 0.2%) and drinking water. All experiments were performed in accordance with the German animal protection law.
Study Design
The present study was comprised of 100 animals, aged 15 months, randomly assigned to 3 groups. Two groups had 45 animals each, and 1 group had 10 animals. The latter group served as controls at the start of the study and was used as described in the interim analysis. The other rats were treated by drinking water that contained either placebo or an antihypertensive dose of ramipril (1 mg · kg⁻¹ · d⁻¹). The treatment commenced immediately after randomization and was adjusted to the actual water consumption. Body weights and systolic BP were determined every 3 months, by use of tail plethysmography. Deaths were recorded as they occurred.

Interim Analysis
The interim analysis was scheduled for the time when >80% of the placebo-treated animals had died, which was after 21 months. Ten animals from each group were randomly selected and anesthetized (hexobarbitone, 80 mg/kg IP). Blood samples were drawn, and thoracic aortas and hearts were removed for molecular, biochemical, and/or functional analyses. Renin activity, and concentrations of aldosterone and angiotensin (Ang) I and II were determined in plasma. ACE activities in plasma, thoracic aortas, and right cardiac ventricles were radioenzymatically measured by use of 3 H-Gly-Gly as substrate (Hycor ACE-activity test).

Isolated Working Heart
The hearts were perfused according to Langendorff’s method with an oxygenated (95% O₂, 5% CO₂) noncirculating Krebs-Henseleit solution of the following compositions (mmol/L): NaCl, 118; KCl, 4.7; CaCl₂, 2.5; MgSO₄, 1.6; NaHCO₃, 24.9; KH₂PO₄, 1.2; glucose, 5.5; Na-pyruvate, 2.0. Perfusion pressure was 60 mm Hg. A catheter placed into the pulmonary artery drained the coronary effluent perfusate that was collected for determination of coronary flow and venous PO₂ measurements. The left atrium was cannulated by an incision of the left auricle. After a 15-minute equilibration period, the heart was switched into the working mode, with the use of a filling pressure (preload) of 15 mm Hg at an afterload pressure of 60 mm Hg. The mechanical performance of the hearts was stable for the whole experimental period of 90 minutes.

Flow and pressure signals for computation were obtained from the PLUGSYS measuring system (Hugo Sachs Elektronik). Computation of data was performed with a sampling rate of 500 Hz, averaged every 2 seconds, by use of the software ACQuire Plus V1.21F (PO-NE-MAH).

For further characterization of pathophysiological reactions of the heart, the external heart power (EHP) was measured and calculated using the formula, EHP = [m · (−1 · g⁻¹ · s⁻¹)] · pressure · volume work + acceleration work/[SV · (MAP - LAP)] + [1/2 · SV · d · (SV/τ + r'))]; HR · g⁻¹ · s⁻¹ · SV indicates stroke volume; MAP, mean aortic pressure; LAP, mean left atrial pressure; d, specific weight perfusate (1.004 g/cm³); r, inner radius of aortic cannula; e, ejection time; HR, heart rate; LV, left ventricle; LVVwt, left ventricular wet weight. The function of the left ventricle was altered by changing the aortic pressure (afterload) at constant left atrial filling pressure (preload). By adjusting the Starling resistance, the aortic outflow could be switched during 1 minute from the fixed baseline afterload to a preset high afterload, producing step-wise rises in mean aortic pressure.

Thereafter, the hearts were gently blotted to dryness, and the perfusate was removed by a 30-minute centrifugation at 4°C (>100 000 g). 100 μg total of the protein extracts were subjected to SDS-PAGE electrophoresis and transferred to nitrocellulose membranes (Hybond, Amersham). The eNOS protein was detected by use of a specific antibody (monoclonal anti–NOS-III, Transduction Laboratories) and visualized by enhanced chemiluminescence with a commercially available kit (Amer sham). As a secondary antibody, an anti-mouse IgG antibody coupled to alkaline phosphatase was used (Jackson ImmunoResearch Laboratories). Chemiluminescence was analyzed and quantified by scanning with a Fluorimag er 595-system (Molecular Dynamics).

Endothelium-Dependent Relaxation in Isolated Rings of the Thoracic Aorta
The method used was the same as previously described.⁵

Measurement of NO-Release
The porphyrinic microsensor (detection limit 10 mmol/L for in vitro NO measurements) was prepared as described previously.⁷,⁸ The amperometric signal at constant potential of 0.67 V was measured with a voltametric analyzer (PAR model 273, Princeton Applied Research) interfaced with an IBM 80466 computer with data acquisition and control software. Linear calibration curves were constructed for each sensor from 2 · 10 mmol/L to 2 · 10 mmol/L NO, before and after in vitro measurements, with the use of aliquots of saturated NO prepared as described.⁹

An opened thoracic aorta ring was immersed in physiological solution and positioned under a dissecting microscope. A porphyrinic sensor was lowered on the surface of the observed blood vessel with the help of a stereotactic micromanipulator. This was indicated by a small (10 pA) and short (ms) piezoelectric signal. Two auxiliary electrodes (platinum and silver/silver chloride) were positioned in a physiological solution near the opened aortic strip. The concentration of NO decreased exponentially with distance and was undetectable at distances >130±20 μm from the endothelium.

Measurement of Superoxide Production
The superoxide (O₂⁻) concentration in aortic tissue was determined by a chemiluminescence method.¹⁰ Each (0.8 to 1.5 mg) tissue sample was placed in 2 mL of HBSS adjusted to pH 7.4, then adequate lucigenin was added to establish a concentration of 2.5 · 100 μmol/L. The generated O₂⁻ was measured after a 2-minute incubation (basal value) by addition of 10 μL of 1.2 · 100 μmol/L A23187. Photons were counted during the first 20 seconds after the addition of A23187 and were calibrated as O₂⁻ concentration by constructing standard curves based on photons emitted by O₂ stoichiometrically generated by reaction of xanthine and xanthine oxidase.¹¹

Statistical Analysis
The data, except survival, are given as mean±SE. ANOVA was used. Tukey’s test was used for post-ANOVA multiple pair comparisons. Cumulative survival was analyzed for differences according to the Kaplan-Meier method, after which, Cox-Mantel log-rank test was used. Null hypotheses were rejected at P<0.05.

Results
Measurements of Body Weight and Systolic BP
The body weights of the rats (370 to 410 g) did not differ over the course of the experiment. At the start of the study when the SHR were 15 months old, they had a systolic BP of 173±4 mm Hg that remained unaltered in the placebo and control group until the end of the study, at 21 months of age. ACE inhibition with ramipril over 6 months significantly decreased systolic BP in SHR to 121±3 mm Hg, which was similar (115±8 mm Hg) to the systolic BP of untreated normotensive WKY.⁶

Cumulative Survival
Placebo-treated SHR began to die from cardiovascular complications at an age of ~15 months and had all died after 21
months. Ramipril treatment at a BP lowering dose of 1 mg·kg\(^{-1}\)·d\(^{-1}\) (at the age of 15 months, when the first deaths of placebo-treated SHR occurred) extended the maximal lifespan of these animals to 30 months (Figure 1). This life expectancy is almost identical to the lifespan of untreated normotensive WKY.\(^5\)

**Interim Analysis after 21 Months**

Heart weights (mg/100 g body wt) of ramipril-treated SHR were significantly less than (total, 463±12; left ventricle, 356±10; right ventricle, 63±2) age-matched placebos (total, 711±21; left ventricle, 511±16; right ventricle, 112±11) and less than controls (total, 707±26; left ventricle, 509±18; right ventricle, 110±9).

Markers of the renin-angiotensin system such as plasma renin activity and plasma Ang I concentration were significantly greater in treated SHR, whereas plasma Ang II and serum aldosterone concentrations and ACE activities in the serum, thoracic aortas, and right cardiac ventricles were significantly lower versus age-matched placebos and controls (Table).

**Markers of the Renin-Angiotensin System in Old SHR After 6 Month ACE-Inhibitor Treatment**

<table>
<thead>
<tr>
<th>Marker of the RAS</th>
<th>Control</th>
<th>Placebo</th>
<th>Ramipril</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRA, ng Ang I · mL(^{-1})· 10 min(^{-1})</td>
<td>2.6±0.2</td>
<td>2.7±0.3</td>
<td>16.4±2.9*†</td>
</tr>
<tr>
<td>Ang II, pg · mL(^{-1})</td>
<td>61±9</td>
<td>67±8</td>
<td>34±5†</td>
</tr>
<tr>
<td>Aldosterone, pg · mL(^{-1})</td>
<td>277±16</td>
<td>284±15</td>
<td>179±9†</td>
</tr>
<tr>
<td>ACEA-Plasma, nmol · ml(^{-1})· min(^{-1})</td>
<td>122±2</td>
<td>119±4</td>
<td>9±2†</td>
</tr>
<tr>
<td>ACEA-Thoracic Aorta, nmol · mg protein (^{-1})· h(^{-1})</td>
<td>1219±59</td>
<td>1299±89</td>
<td>86±11†</td>
</tr>
<tr>
<td>ACEA-Right Cardiac Ventricle, nmol · mg protein (^{-1})· h(^{-1})</td>
<td>72±10</td>
<td>76±11</td>
<td>12±2†</td>
</tr>
</tbody>
</table>

*P<0.05 vs placebo, †P<0.05 vs control, n=10 per group.

**Expression and Activity of eNOS**

Densitometric analysis of Western blots showed a significant increase (=5-fold) of the expression of eNOS in the left cardiac ventricles of ramipril-treated animals versus controls and placebo-treated SHR (Figure 4). The expression of eNOS in control animals was not significantly different from placebo-treated SHR.

Long-term ACE inhibition with ramipril treatment increased the release of NO from freshly excised thoracic aortic rings (Figure 5A). NO released from the aortas of controls and placebo-treated SHR, after stimulation with the receptor independent calcium ionophore A23187, was 335±18 nmol/L and 340±20 nmol/L, respectively. Calcium ionophore-stimulated NO concentration increased by 47% (to 500±10 nmol/L) in aortas of ramipril treated SHR. Under the same conditions, age-matched untreated normotensive WKY showed an aortic NO release of 580±40 nmol/L. Both the basal and total O\(_2\)\(^{-}\) concentrations (observed before and after

**Figure 1.** Effect of late treatment with ramipril on cumulative percent survival in SHR. • indicates placebo; ▲, antihypertensive dose of ramipril (1 mg · kg\(^{-1}\)· d\(^{-1}\)). Percentage survival in SHR was significantly enhanced with ramipril treatment vs placebo (Kaplan-Meier analysis followed by Cox-Mantel log-rank test, ramipril) \(\chi^2=48.44, P<0.05\).

**Figure 2.** External heart work in isolated working hearts from SHR. ■ indicates 15-month-old SHR at the beginning of the study (control); ●, placebo; ▲, ramipril (1 mg · kg\(^{-1}\)· d\(^{-1}\)) treated SHR 6 months later (interim analysis at the age of 21 months). *P<0.05 vs placebo, #P<0.05 vs control, n=10 per group.

**Isolated Working Heart Preparation**

Extracellular heart relaxation in response to acetylcholine was strongly impaired in potassium chloride precontracted aortic rings from control animals (15-months-old) as well as from placebo-treated SHR (21-months-old). This impaired relaxation was significantly reversed by ramipril treatment (Figure 3).

**Isolated Thoracic Aorta**

Endothelium-dependent relaxation in response to acetylcholine was strongly impaired in potassium chloride precontracted aortic rings from control animals (15-months-old) as well as from placebo-treated SHR (21-months-old). This impaired relaxation was significantly reversed by ramipril treatment (Figure 3).

**Figure 3.** Endothelium-dependent relaxation by acetylcholine expressed as percentage reversal of potassium chloride (20 mmol/L) elicited contractions in intact aortic rings from SHR at interim analysis. ■ indicates control group after 15 months (start of the study); ●, placebo-treatment group after 15 months; ▲, ramipril (1 mg · kg\(^{-1}\)· d\(^{-1}\)) treatment group after 21 months. *P<0.05 vs placebo, #P<0.05 vs control, n=10 per group.
stimulation of NO release with calcium ionophore) decreased slightly, but nonsignificantly, after ramipril treatment versus controls and placebo-treated animals (Figure 5B).

Discussion

The present study documents that late treatment with the ACE inhibitor ramipril in a BP lowering dose (1 mg \( \cdot \text{kg}^{-1} \cdot \text{d}^{-1} \)) extended life expectancy of SHR by 43%, which corresponds to the lifespan of normotensive placebo-treated WKY.6 The prime effect that contributes to this lifespan extension is, presumably, the normalization of BP followed by restoration of the endothelial function. Ramipril treatment of old SHR restored NO formation by the endothelium to a level of 500 \( \pm 10 \) nmol/L, which is similar to the level observed for untreated normotensive WKY rats. The restoration of NO production by the endothelium preceded a BP reduction with regression of left ventricular hypertrophy and improved heart function. The capability of the endocardium and the cardiac endothelium to produce optimal concentration of NO through changes in mechanical forces during each systolic and diastolic period of cardiac contraction is crucial in maintaining proper heart function.12 In addition to NO restoration, many other regulators, such as sympathetic activity,13 endothelin1, and thromboxane A2,14 could be positively affected by inhibition of the renin-angiotensin system.

Because an activated renin-angiotensin system is implicated in the development and maintenance of hypertension and cardiac hypertrophy,15 it is not surprising that late ramipril treatment resulted in a regression of established myocardial hypertrophy, which is also reflected in improved cardiac function. However, the possibility cannot be excluded that the heart weight differences were caused by different interstitial and/or intracellular fluid volumes in the myocytes that were positively influenced by ramipril treatment. In addition to the regression of left ventricular hypertrophy, improved function in the rat heart could also be mediated by endothelium-derived kinins.16,17

An increase in bioavailability of NO in ramipril-treated animals seems to be caused by the increase of eNOS expression and not by the decrease of \( \text{O}_2^- \) production. This is in contrast to our prevention study, in which, after early, lifelong ramipril treatment, we found reduced \( \text{O}_2^- \) production, in addition to the upregulation of eNOS, in aortas of old SHR.6 The concentration of NO is significantly higher in the cardiovascular system of untreated WKY than in untreated SHR. This difference is caused by a much lower production of \( \text{O}_2^- \) in WKY than in SHR. The porphyrinic sensor only detects the net NO concentration (NO that is not consumed in fast chemical reaction with \( \text{O}_2^- \) and can freely diffuse to a target cell). This net NO concentration depends both on the expression of eNOS and on the accumulation of \( \text{O}_2^- \).18 Preventive long-term treatment of hypertensive rats with ramipril6,6 showed similar results to those of the present study. However, late treatment, in contrast with early treatment, with ramipril did not decrease generation of \( \text{O}_2^- \).

Thus, it can be assumed that in SHR late ramipril treatment upregulated eNOS leading to a higher NO availability, which, in turn, reversed the impaired endothelium-dependent vasodilation. This contributed to the lifespan extension of old hypertensive rats. Similar data were obtained with only a
3-month treatment of old SHR with the Ang II subtype AT₁ receptor blocker losartan. It is conceivable that the ramipril-induced inhibition of the endogenous breakdown of kinins mediated the increase in survival, because co-treatment with the bradykinin receptor antagonist icatibant suppressed the enhanced aortic cGMP content after high- and low-dose treatment with ramipril. Furthermore, an upregulated eNOS was found in cultured endothelial cells incubated for 48 hours with 8-bromo-cGMP. Another candidate that contributes to the upregulation of eNOS expression induced by endogenous kinins might be cAMP. Increased endothelial cAMP is induced by forskolin and amplified by activation of a cAMP-dependent protein kinase, the bradykinin-induced synthesis of cGMP. Recently, upregulation of eNOS by ACE inhibition was also described in the human arial myocardium.

**Conclusion and Clinical Implications**

Results of the present study show that different beneficial effects of late antihypertensive ACE-inhibitor treatment on major cardiovascular functions might contribute to the lifespan extension of rats with a genetic form of hypertension. The pivotal beneficial effects of ACE inhibition are BP reduction and restoration of endothelial function. This is manifested by a significantly greater NO formation, which is also crucial for normal BP, the regression of cardiac left ventricular hypertrophy, and the improvement of cardiac function. Various molecular/biochemical mechanisms can be attributed to these effects. Suppression of tissue ACE expression and activity decreases (1) local Ang II concentrations, which are mainly related to antihypertensive and antihypertrophic actions, and (2) increased local concentrations of kinins which may preserve cardiac functions. In addition, the upregulation of eNOS associated with subsequently enhanced NO availability provides functional restoration of the endothelium. It remains to be confirmed by the outcome of long-term preventive ACE-inhibitor treatment of high-risk patients with myocardial infarction and coronary artery disease whether these beneficial effects observed in SHR, in particular the extended life expectancy, are also achievable in humans.

**Acknowledgments**

This work was supported in part by grants from Hoechst Marion Roussel, Inc. The Public Health Service (HL-55397), and the Research Excellence Fund, Center for Medical Research, Oakland University.

**References**

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Hypertension. 1999;34:291-295
doi: 10.1161/01.HYP.34.2.291

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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