Decreased susceptibility of cardiac function to hypoxia-reoxygenation in renin-angiotensinogen transgenic rats

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Wagner, Kay-Dietrich, Vanja Essmann, Karsten Mydlak, Manfred Wirth, Gunnar Gmehling, Jürgen Bohlender, Harald M. Stauss, Joachim Günther, Ingolf Schimke, and Holger Scholz. Decreased susceptibility of cardiac function to hypoxia-reoxygenation in renin-angiotensinogen transgenic rats. Am J Physiol Regulatory Integrative Comp Physiol 283: R153–R160, 2002. First published February 14, 2002; 10.1152/ajpregu.00491.2001.—We tested the hypothesis that the renin-angiotensin system (RAS) protects the contractile function of the myocardium against the damaging effect of hypoxia-reoxygenation. For this purpose, the contractility of isolated papillary muscles from wild-type (WT) rats and from rats expressing human renin and angiotensinogen as transgenes (TGR) was compared. After 15 min of hypoxia, peak force (PF) was decreased to 24 ± 5% of the normoxic values in TGR (n = 10) and to 18 ± 1% in WT rats (n = 12). PF and relaxation rates recovered completely in TGR but not in WT rats during 45 min of reoxygenation. Improved contractility of the papillary muscles from TGR during hypoxia-reoxygenation correlated with increased glutathione peroxidase activities and creatine kinase (CK)-MB and CK-BB isoenzyme levels. On the other hand, inhibition of the RAS with ramipril (1 mg/kg body wt for 3 wk) in WT animals resulted in deterioration of the contractile function of the papillary muscles during reoxygenation compared with untreated rats. These findings suggest that activation of the RAS protects contractile function of the cardiac muscle against hypoxia-reoxygenation, possibly through changes in CK isoenzymes and enhanced antioxidant capacity.

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In view of these controversies, we aimed to further analyze the role of the RAS in the contractile response of the myocardium to ischemia-reperfusion. For this purpose, we took advantage of a recently developed rat line that harbors the human renin and angiotensinogen transgenes (7). Both transgenes are expressed in the hearts of TGR rats, which exhibit severe ANG II-dependent arterial hypertension and cardiac hypertrophy (7). Compared with pharmacological stimulation of the RAS, adverse drug effects can be avoided with the use of renin-angiotensinogen double-transgenic animals. As the major finding of this study, we report that mechanical function during hypoxia-reoxygenation is better preserved in myocardial tissue from TGR than from wild-type (WT) rats.

METHODS

Animal experiments and preparation of rat hearts. This investigation conforms to the guidelines for the care and use of laboratory animals published by the National Institutes of Health [DHHS Publication No. (NIH) 85-23, revised 1996]. The transgenic male Sprague-Dawley rats used in this study (TGR, n = 10) carried the complete human genomic angiotensinogen gene with 1.6 kb of 5'-flanking region and 3.5 kb of 3'-flanking sequence, as well as the entire human renin gene with 10 exons and 9 introns, including 3 kb of upstream promoter region and 1.2 kb of downstream sequence (7). Male normotensive age-matched WT Sprague-Dawley rats served as controls (n = 12). Arterial blood pressure was measured in conscious rats with a catheter that was inserted into the femoral artery and connected to a pressure transducer (DTX Plus, Ohmeda), as described in detail elsewhere (38). On the day of the experiments, the rats were anesthetized with ether, and their hearts were quickly excised. After removal of the hearts from the animals, the left and right ventricles were weighed separately. Tissue specimens from the left and right ventricular free walls were snap frozen in liquid nitrogen for biochemical analyses. Thereafter, the left ventricular posterior papillary muscles were dissected. In some experiments, RAS inhibition was achieved by feeding rats the ACE inhibitor ramipril for 3 wk (n = 12). For this purpose, the daily dose of ramipril (1 mg/kg body wt) was given to the rats with the drinking water. Untreated age-matched animals served as controls.

Experimental protocol for papillary muscles. Isometric contractions of isolated papillary muscles were set up in a tissue bath, as described in detail elsewhere (17, 42, 43). Briefly, the bathing solution consisted of (in mM) 140 NaCl, 5.0 KCl, 1.5 CaCl₂, 1.1 MgCl₂, 10.0 Tris-HCl, and 11.1 glucose, pH 7.45, and was adjusted to 31°C with a thermocontrolled, recirculating water bath. The extracellular solution was bubbled in a O₂-sensitive electrode immersed in the bathing solution was (17). To avoid irreversible functional damage of the papillary muscles, hypoxic exposure was limited to 15 min (17, 42). An O₂-sensitive electrode immersed in the bathing solution was used to monitor O₂. Field stimulation of the papillary muscles at a frequency of 0.5 Hz and isometric force measurements were performed using the stimulator module, the force transducer, and the bridge amplifier of the Plugsys system 603 (Hugo Sachs Elektronik, March-Hugstetten, Germany). Data acquisition, analysis of the mechanogram, and statistics were performed on a personal computer with software developed in our laboratory (42). Preload was adjusted to 50% of the value causing maximum load-dependent peak force (PF). Under these conditions, force development was nearly stable for >2 h, suggesting that the mechanical function of the preparations was preserved. Biphasic rectangular impulses of 7-ms duration and a current 30% above threshold were applied. To test whether the O₂ supply to the preparations was adequate, a fourfold increased stimulation frequency was applied for several minutes. This maneuver did not impair contractile function, suggesting that tissue oxygenation was sufficient. To avoid a hypoxic core in the papillary muscles, the preparations were equilibrated for 25 min at low stimulation frequency of 0.25 Hz in the presence of 0.75 mmol/l CaCl₂ and low preload of 1 mN. The papillary muscles were allowed to recover during reoxygenation with pure O₂ until no further increase in PF was observed. PF, the time to PF (TPF), and the relaxation time for the decline of force by 50% and 97% (RT50 and RT97, respectively) were determined from the force signal. TPF was calculated as the time interval between the initial rise in active force and maximum force development. RT50 and RT97 were determined as the time elapsing between PF and the decline of force by 50% and 97% of PF, respectively. Maximum (dF/dₜ₉₅₀) and minimum (dFs/dₜ₉₇₇₇) values were calculated from the dF/dₜ signal. After the experiments, the length and the weight of the papillary muscles were measured, and the preparations were subsequently snap frozen in liquid nitrogen for biochemical measurements. Because only very small tissue samples were available from the papillary muscles, the biochemical analyses were limited to determination of GSH-Px activities and heat shock protein (HSP) content. The volume (V) of the papillary muscles was estimated as follows: V = πr²h, where r is radius and h is the length of the preparations. With the assumption that density of the cardiac tissue is 1 g/ml, the mean cross-sectional area of the preparations was calculated as follows: πr² = V/h. The parameters of mechanical function of the papillary muscles were expressed per cross-sectional area.

Sample preparation for measurement of creatine kinase isoenzyme activities and antioxidant enzymes. Tissue samples were homogenized three times for 15 s in a 20-fold volume of ice-cold 150 mmol/l KCl containing 0.01% butylated hydroxyanisole with an Ultraturrax homogenizer at 75% of its maximum speed.

Determination of SOD and GSH-Px activities. Total SOD (t-SOD) activities were measured according to the protocol of Beauchamp and Fridovich (4) using the RANSOD-kit (Randox Laboratories). This method is based on the ability of t-SOD to prevent the formation of formazan from 2-(4-iodophenyl)-3-(4-nitro)-5-phenyltetrazolium chloride by superoxide radicals generated by xanthine oxidase/xanthine (4). The formation of formazan was recorded spectrophotometrically (model UV-2101 PC, Shimadzu) at 550-nm wavelength. Inhibition by 50% of the 2-(4-iodophenyl)-3-(4-nitro)-5-phenyltetrazolium chloride reduction after addition of the sample (0.01–0.02 mg tissue/ml test volume) was defined as 1 U of SOD. Mn-SOD activity was measured as described above in the presence of 2 mmol/l cyanide to inhibit CuZn-SOD activity, which was calculated on the basis of the activities of t-SOD and Mn-SOD. GSH-Px activity was determined according to Paglia and Valentine (30) using the RANSEL kit (Randox Laboratories).

Measurement of creatine kinase isoenzyme activities. Total creatine kinase (CK) activity was measured with the CK-NAC Actv Kit (Roche Biochemicals). Forty micrograms of tissue were used per milliliter of test volume. The CK isoenzyme pattern was determined according to Laser et al. (18) using the rapid electrophoresis system as a separation unit.
For agarose gel electrophoresis, we used the rapid electrophoresis system CK15 isofoms kit (Rolf Greiner Bio-Chemica). Protein concentrations were measured according to Lowry et al. (22).

Western blot analysis of HSPs. Immunoblotting of HSP25 and HSP72 was performed according to the method of Lutsch et al. (23) using the following antibodies: 1) polyclonal rabbit anti-HSP25 antibody (37) and 2) monoclonal mouse anti-HSP72 antibody, clone C92F3A-5 (StressGen). Proteins were visualized with alkaline phosphatase-conjugated secondary antibodies (Sigma). The optical densities of the immunoreactive bands were evaluated by integration over the whole area of extinction with a laser densitometer (Ultrascan model 2202, LKB). The DC protein assay (Bio-Rad) was used for protein determination.

Statistical analysis. Values are means ± SE. Intergroup comparisons for biochemical measurements were done using a one-way analysis of variance with the Bonferroni test as post hoc test. For measurements of papillary muscle contractility, the Wilcoxon test of unpaired or paired samples was used. P < 0.05 was considered statistically significant.

RESULTS

Body and heart weights of TGR and WT rats as well as the ramipril-treated animals and their untreated controls are shown in Table 1. The heart weight-to-body weight ratios were significantly increased (+32%) in TGR vs. age-matched WT rats, indicating cardiac hypertrophy. Interestingly, the elevated overall heart weight-to-body weight ratios in TGR were accounted for by the higher (+41%) left ventricular weight indexes, and not by the right ventricular weight-to-body weight ratios, which were similar in both groups. Systolic arterial blood pressure values were 135 ± 1 mmHg (n = 12) and 226 ± 15 mmHg (n = 10) in WT rats and TGR, respectively (P < 0.01). Diastolic blood pressure values were 80 ± 1 and 149 ± 10 mmHg in WT rats and TGR, respectively (P < 0.01).

Contractile function of isolated papillary muscles. Contractile function of the left ventricular papillary muscles was compared between TGR and WT rats. At normoxia, PF development of the isometrically contracting preparations was similar in both groups. The rates of contraction and relaxation were also not significantly different between the papillary muscles from TGR and WT animals (Table 2).

Mechanical properties of the isolated papillary muscles were considerably altered during restricted O₂ supply. Hypoxia caused a strong decrease of PF in both groups (Fig. 1A), which, however, was less pronounced in TGR than in WT rats during the initial phase of hypoxia. After 15 min of hypoxia, PF was reduced to ~17% and 24% of the normoxic values in WT rats and TGR, respectively. The marked decline in PF during hypoxic exposure was associated with a more rapid force development, as suggested by the shortened TPF in both groups (Fig. 1C). Rates of contraction and relaxation were reduced in both groups during hypoxia, but this effect was weaker in TGR than in WT rats (Fig. 1, B and D). Altered relaxation rates during restricted O₂ supply are indicated by the delayed fall of PF by 50% (RT₅₀) and 97%, respectively (Fig. 1, E and F).

The mechanical function of the preparations from TGR recovered almost completely during reoxygenation (Fig. 1). In contrast, after 45 min of reoxygenation, PF of the papillary muscles from WT animals reached only 68% of the normoxic values (Fig. 1A). Recovery of the rates of contraction and relaxation was also better preserved in TGR than in WT preparations (Fig. 1, B and D). Addition of ANG II to the bathing solution at a final concentration of 100 nmol/l had no significant effect on the contractile function of the isolated papillary muscles (data not shown). For comparison, ANG II used at the same concentration of 100 nmol/l caused contractions of isolated segments of rat aorta (unpublished observations).

We also examined whether the contractile function of the myocardium would be affected by chronic inhibition of the RAS in normal rats. For this purpose,
male Sprague-Dawley rats (n = 12) received ramipril (1 mg·kg body wt⁻¹·day⁻¹) for 3 wk with the drinking water, and isolated papillary muscles from these rats were studied. Ramipril treatment impaired the contractility of the papillary muscle preparations during hypoxia-reoxygenation. For comparison, PF after 45 min of reoxygenation was 26 ± 3% and 46 ± 6% of the prehypoxic values in ramipril-fed and untreated rats, respectively (Fig. 2A). The maximum rates of contraction (dF/dt_max) were 23 ± 3% of the normoxic values in ramipril-treated rats compared with 43 ± 5% in control rats after 45 min of reoxygenation. Similarly, the maximum rates of relaxation (dF/dt_min) were 20 ± 3% and 40 ± 1% of the prehypoxic values with and without RAS inhibition, respectively.

**Antioxidant enzymes and HSPs.** To examine whether the differences in contractile function between TGR and WT rats were related to changes in antioxidant enzymes, we measured SOD and GSH-Px activities in cardiac tissue from both groups. In TGR, the activity of GSH-Px was increased in the left and right ventricular tissue (405 ± 17 and 413 ± 15 mU/mg, respectively) compared with WT rats (354 ± 11 and 338 ± 11 mU/mg, respectively; Fig. 3). No significant differences in GSH-Px activities could be detected between the left and right ventricles in both groups. In addition, in the papillary muscles, GSH-Px activities were higher in TGR than in WT rats: 292 ± 32 and 194 ± 8 mU/mg, respectively (P < 0.05). On the other hand, inhibition of the RAS by administration of ramipril for 3 wk significantly reduced GSH-Px activities in the left ventricular tissue of normal rats: 326 ± 21 vs. 396 ± 18 mU/mg in untreated animals (Fig. 2B). SOD activities were similar in the left and right ventricular homogenates of TGR and WT rats (Fig. 3).

HSPs have been implicated in the myocardial response to stress, including ischemic injury (8, 12, 28). We therefore examined whether improved contractile function of the TGR during hypoxia-reoxygenation could be due to enhanced myocardial expression of protective HSPs. However, as shown in Fig. 4, HSP72 levels in the right and left ventricles of the TGR were not significantly different from those in normal rats. HSP72 expression was also not significantly different.

![Fig. 1. Contraction and relaxation parameters of papillary muscles from transgenic (TGR, n = 10) and wild-type rats (WT, n = 12) during 15 min of hypoxia followed by 45 min of reoxygenation. Data were normalized to normoxic control values at 0 min. PF, peak force; dF/dt max, maximum rate of contraction; TPF, time to peak force; dF/dt min, maximum rate of relaxation; RT 50, relaxation time for the decline of PF by 50%; RT 97, relaxation time for the decline of PF by 97%. *P < 0.05 vs. WT.](http://ajpregu.physiology.org/)

**R156 CARDIAC CONTRACTILITY IN TGR**

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between the papillary muscles of TGR and WT rats: 92 ± 10% and 100 ± 13%, respectively. In contrast, expression of the small HSP, HSP25, was significantly enhanced in the left ventricles (137 ± 10% and 100 ± 4% in TGR and WT, respectively, \( P < 0.05 \)), but not in the right ventricles (86 ± 11% and 100 ± 12% in TGR and WT, respectively) of the TGR (Fig. 4). A higher content of HSP25 was also measured in the left ventricular papillary muscles of TGR: 198 ± 32% and 100 ± 6% in TGR and WT rats, respectively \( P < 0.05 \).

**CK isoenzymes.** We and others previously reported that improved contractile function of the hypertrophied myocardium during hypoxia-reoxygenation correlates with redistribution of cardiac CK isoenzymes (27, 33, 34, 41). In this study, we found that total CK activity was reduced in the left ventricular homogenates from TGR compared with age-matched WT rats: 14.3 ± 0.7 vs. 16.2 ± 0.5 U/mg protein \( P < 0.05 \). In contrast, the right ventricular total CK activities were similar in both groups: 15.4 ± 0.7 and 16.5 ± 0.6 U/mg in TGR and WT rats, respectively (Table 3). The fraction of the “adult” CK isoenzyme CK-MM was significantly reduced in the left ventricular tissue of TGR (56.8 ± 0.8%) compared with WT rats (59.2 ± 0.7%, \( P < 0.05 \)) and also compared with the nonhypertrophied right ventricles (59.8 ± 0.6%, \( P < 0.05 \); Table 3). The fractions of the “fetal” CK isoforms CK-MB (16.5 ± 0.6% and 14.0 ± 0.5% in TGR and WT, respectively, \( P < 0.05 \)) and CK-BB (1.5 ± 0.1% and 1.2 ± 0.1% in TGR and WT, respectively, \( P < 0.05 \)) were significantly increased in the hypertrophied left ventricles of TGR vs. WT rats but unchanged in the right ventricular tissue (Table 3).

**DISCUSSION**

Even though the importance of the RAS in cardiac muscle physiology is widely recognized, its precise role in ischemia-reperfusion injury of the heart has remained unresolved. In this study, we report for the first time that transgenic expression of human renin and angiotensinogen genes in rats protects the mechanical function of the myocardium during hypoxia and subsequent reoxygenation. Hypoxia-reoxygenation is considered a major constituent of ischemia-reperfusion injury of the heart. Despite activation of the RAS in the transgenic rats (7), cardiac hypertrophy was seen in the left but not in the right ventricle of TGR. It remains to be determined whether the right ventricular myocardium is less susceptible to the growth-promoting effect of ANG II or whether the hypertrophic response of the right ventricles to enhanced RAS activity is neutralized by an unknown secondary mechanism(s).

Similar to our results, a shift in the CK isoenzyme pattern to a more fetal phenotype with increased CK-MB and CK-BB fractions has been reported in different animal models of enhanced cardiac growth (27, 33, 34, 41). The changes in cardiac CK isoenzymes were seen in the left but not in the right ventricular tissue of the TGR. Similarly, cardiac hypertrophy was restricted to the left ventricle of TGR, suggesting that the changes in CK isoenzymes were related to enhanced cardiac growth, rather than direct activation by the RAS. This interpretation is supported by a recent study reporting upregulation of the CK B gene in overload-induced cardiac hypertrophy that was insensitive to ACE inhibition (34).

Activity of the antioxidant enzyme GSH-Px was elevated above normal in the left and right ventricles of the TGR, suggesting that GSH-Px in the myocardium is controlled by the RAS and not simply related to cardiac hypertrophy. In contrast, SOD activities were not significantly different between left and right ventricular tissue from WT rats and TGR. This finding is seemingly in conflict with the results obtained with other models of cardiac hypertrophy. For example, a decrease in cardiac SOD activity has been reported in spontaneously hypertensive rats (41), whereas SOD activity was increased during postinfarction hypertrophy (42) and lowered (6) or elevated (10) after aortic banding. These data and our present findings suggest that SOD activity is regulated by a complex network of
signaling mechanisms and not directly related to RAS activity.

To explore whether the observed changes in cardiac muscle biochemistry could be relevant for the mechanical function of the myocardium, we compared the contractility of papillary muscles from the hypertrophied left ventricles of TGR and WT rats. At normoxia, no significant differences in the mechanical properties were observed between TGR and WT animals. The rapid fall in PF shortly after the onset of hypoxia can be explained by a decrease in the $Ca^{2+}$/sensitivity of the myofilaments likely due to accumulation of inorganic phosphates resulting from rapid breakdown of energy-rich phosphates (2, 5). Similar to the findings in the TGR, the hypertrophied papillary muscles from chronically infarcted rat hearts responded to hypoxia with a delayed and less pronounced decrease in PF (42). The exact mechanism for this phenomenon is not fully understood. However, improved contractility of the myocardium can result from a very rapid breakdown of creatine phosphate (1), which may cause in-

![Fig. 3. Activities of antioxidant enzymes GSH-Px and different superoxide dismutase (SOD) isforms as well as total SOD (t-SOD) activity in left (LV) and right ventricular (RV) tissue from transgenic (n = 10) and WT rats (n = 12). Values are means ± SE. *P < 0.05 vs. WT.](image)

![Fig. 4. Expression of 25- and 72-kDa heat shock proteins (HSP25 and HSP72) in left and right ventricular cardiac tissue homogenates from transgenic (n = 10) and WT rats (n = 12). Values are means ± SE. *P < 0.05 vs. WT. †P < 0.05 vs. RV.](image)

Table 3. Activities of CK isoenzymes in LV and RV homogenates from TGR and WT rats

<table>
<thead>
<tr>
<th>Group</th>
<th>TGR (n = 10)</th>
<th>WT (n = 12)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>LV</td>
<td>RV</td>
</tr>
<tr>
<td>CK-MM</td>
<td>56.8 ± 0.8*†</td>
<td>59.8 ± 0.6</td>
</tr>
<tr>
<td>CK-Mi</td>
<td>24.9 ± 0.6</td>
<td>24.4 ± 0.7</td>
</tr>
<tr>
<td>CK-MB</td>
<td>16.5 ± 0.6*†</td>
<td>14.4 ± 0.6</td>
</tr>
<tr>
<td>CK-BB</td>
<td>1.5 ± 0.1*</td>
<td>1.4 ± 0.1</td>
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Values are means ± SE expressed as percent; n, number of rats. CK, creatine kinase. *P < 0.05 vs. WT. †P < 0.05 vs. RV.
tracellular alkalization (15). In addition to the enhanced turnover rates of creatine phosphate, the rise of left ventricular CK-MB and CK-BB isoforms in the TGR may activate phosphoryl transfer from creatine phosphate to ATP (18) because of the lower Michaelis-Menten constants of the CK-MB and CK-BB isoenzymes (44). The shift of CK isoenzymes in hypertrophied cardiac tissue may thereby contribute to the lower sensitivity of the TGR to reduced O2 supply (42).

Similar to the results obtained with hypertrophied myocardium after infarction (42), PF development and the rates of contraction and relaxation recovered better from hypoxia in the papillary muscles of TGR than WT rats. On the other hand, RAS inhibition with ramipril impaired the mechanical function of the papillary muscles during reoxygenation and decreased GSH-Px activities. Reduced hypoxic but not ischemic injury was previously observed in hypertrophied rat hearts after banding of the aortic arch (2). It appears possible, therefore, that enhanced cardiac growth is frequently associated with decreased susceptibility of the myocardium to hypoxia-reoxygenation. Thus we assume that the beneficial effect of RAS activation on the contractile function of papillary muscles from TGR during reoxygenation was, at least in part, secondary to cardiac hypertrophy.

Membrane damage during reoxygenation is caused by ROS when the endogenous antioxidant capacity is exhausted (for review see Ref. 9). Enhanced GSH-Px activity, which was found to protect the myocardium against hypoxia-reoxygenation injury in different models of hypertrophy (16, 42), may contribute to improved antioxidant reserve in the TGR. Furthermore, the elevated left ventricular HSP25 levels may exert an additional protective effect during hypoxia-reoxygenation (for review see Refs. 11 and 13).

In summary, our findings demonstrate that the susceptibility of the hypertrophied myocardium to the damaging effect of hypoxia-reoxygenation is decreased in TGR. Changes in the CK isoenzyme pattern, enhanced HSP25 expression, and increased GSH-Px activity may be important for this functional adaptation.

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REFERENCES
25. Mehta JL, Chen H, Li D, and Phillips MI. Modulation of myocardial SOD and iNOS during ischemia-reperfusion by an-
tisense directed at ACE mRNA. J Mol Cell Cardiol 32: 2259–2268, 2000.


