Accelerated Mitochondrial Adenosine Diphosphate/Adenosine Triphosphate Transport Improves Hypertension-Induced Heart Disease

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**Background**—Strong evidence suggests that mitochondrial malfunction, which leads to disturbed energy metabolism and stimulated apoptosis, is a linchpin in the induction and manifestation of cardiac failure. An adequate exchange of ATP and ADP over the inner mitochondrial membrane by the adenine nucleotide translocase (ANT) is thereby essential to guarantee the cellular energy supply.

**Methods and Results**—To explore the effect of an ameliorated mitochondrial ATP/ADP transportation on cardiac dysfunction, we generated transgenic rats overexpressing ANT1 in the heart (ANT rats) and crossed them with renin-overexpressing rats (REN rats) suffering from hypertension-induced cardiac insufficiency. Cardiac-specific ANT1 overexpression resulted in a higher ATP/ADP transportation and elevated activities of respiratory chain complexes. Increased ANT activity in double-transgenic (ANT/REN) animals did not influence excessive hypertension seen in REN rats. Hypertension-induced cardiac hypertrophy in the REN rats was prevented by parallel ANT1 overexpression, however, and left ventricular function remarkably improved. The ANT1 overexpression led to a reduction in fibrosis and an improvement in cardiac tissue architecture. Consequently, the survival rate of ANT/REN rats was enhanced. Further investigations into the cardioprotective mechanism of ANT1 overexpression revealed improved mitochondrial structure and function and significantly reduced apoptosis in ANT/REN rats, shown by lowered cytosolic/mitochondrial cytochrome c ratio, reduced caspase 3 level, and prevented DNA degradation.

**Conclusions**—Myocardial ANT1 overexpression protects against hypertension-induced cardiac pathology. Thus, the improvement in mitochondrial function may be a basic principle for new strategies in treating heart disease.

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**Key Words:** apoptosis ■ heart diseases ■ hypertension ■ hypertrophy ■ remodeling ■ renin ■ survival

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The adenine nucleotide translocase (ANT) is the only known carrier that facilitates the transport of ADP and ATP across the inner mitochondrial membrane.1 The dimeric ANT complex of rodents is nuclear encoded by 3 distinct genes (ANT1, ANT2, and ANT4) that are coexpressed in tissue-specific patterns.2-3 ANT1 is the predominant isoform in the heart. In addition to its role in linking mitochondrial energy production and cytosolic energy consumption, ANT is a binding partner of proapoptotic and antiapoptotic proteins,4 transcription factors,5 and cell-signaling proteins6 and thus is involved in the regulation of apoptosis and intracellular communication.

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Reduced ANT activity and altered ANT isoform expression are observed in human heart failure resulting from various causes7,8; thus, an ANT deficit appeared to contribute to the pathogenesis of myocardial remodeling and cardiac insufficiency. This is supported by the analysis of ANT1 knockout mice that showed characteristics of hypertrophic cardiomyopathy and mitochondrial myopathy.9 Consequently, an increase in the efficiency of ATP/ADP exchange by ANT1 overexpression may improve myocardial function in failing hearts, supporting the hypothesis that ANT eleva-
tion and activation are involved in a cardioprotective program to adapt to cardiac stress before profound myocardial damage occurs.

To test this hypothesis, we generated transgenic rats specifically overexpressing ANT1 in the heart (ANT rats) and crossed them with the transgenic rat strain TGR(mREN2)27 harboring the murine Ren-2 gene. Renin is initially responsible for generating the vasoconstrictor angiotensin II from angiotensinogen, and its permanent overexpression leads to elevated angiotensin levels and consequently to fulminant hypertension combined with cardiac hypertrophy and malfunction.

Methods

Generation of Transgenic Animals

All animal studies were performed according to national guidelines and approved by the institutional animal care committee. The transgenic rat strain with cardiac overexpression of rat ANT1 (TGRMHCrANT1 [ANT]) was developed using the rat αMHC promoter (αMHC; RATMYHAB2; gi:205560; 1 to 1040 bp).13 Rat ANT1 cDNA was amplified by polymerase chain reaction from reverse-transcribed cardiac rat RNA and subcloned into the multicloning site of pCRTM3 TA vector (Invitrogen, Karlsruhe, Germany). After removal of the cytomegalovirus promoter of the plasmid by restriction enzyme digestions, the rat αMHC promoter was inserted between KpnI and BamHI into the multiple cloning site of the vector. This generated recombinant plasmid pCR3pMHC-ratANT1 was digested to a 2.7-kb DNA fragment by HgalI restriction (Figure 1A), which was then microinjected into the pronuclei of single-cell fertilized rat embryos of the Sprague-Dawley background (bred inhouse) to generate transgenic rats.

Southern Blot

ANT1 transgene integration has been proved by Southern blot analysis. For founder detection, 10 μg chromosomal DNA of the potential founders was digested with HindIII. To detect copy number, 5, 10, and 20 μg wild-type (WT) DNA (Sprague Dawley) were digested by EcoRI/HindIII and used for Southern blot (right). Representative RNase protection assay showing ANT1 transgene mRNA expression in different organs of 1 negative (−) and 2 positive (+) male rats (age, 3 months). D, Left, Representative RNase protection assay showing ANT1 transgene mRNA expression in LVs of 2 transgenic (+) and 2 WT (−) rats at an age of 2 days (2d) or 2 months (2m). Right, Quantification of ANT1 transgene expression in LVs of 2-day-old (2d), 2-month-old (2m), 4-month-old (4m), and 8-month-old old rats after autoradiographic signal analysis. Data are shown as multiples after normalization to rL32 mRNA levels (n=6). E, Quantification of ANT protein from isolated LV mitochondria of ANT transgenic and WT rats at an age of 4 or 8 months. n=6 per group. At indicates positive atrium as control; le vent, LV; rt vent, right ventricle; yeast +, yeast plus RNase; yeast −, yeast without RNase; I, transgenic ANT mRNA band; and II, the αMHC mRNA. **P<0.01 vs WT.

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RNase Protection Assay

Total RNA was isolated as described before.15 A 249-bp fragment of pMHC-ratANT1 harboring 183 bp of the αMHC promoter and 66 bp of exon 1 of the AN1 cDNA was subcloned into the T-vector (Promega, Mannheim, Germany). A SP6 polymerase transcribed a radioactive probe complementary to the 249-bp transgenic mRNA.
RNA complementary to 127 nucleotides of the rL32 mRNA was used as positive controls. Using the Ambion Rnase Protection Assay II kit (AMS Biotechnology, Whitney, United Kingdom), we hybridized 20 μg of each RNA sample with ~50 000 cpm of ANT1 and 50 000 cpm of rL32-ribolabeled antisense probe in the same reaction. The hybridized fragments protected from RNaseA+T1 digestion were separated and detected as described before.15 Quantitative analysis was performed by measuring the intensity of the ANT1 bands normalized by the intensity of rL32.

Pressure-Volume-Loop Measurement

In anesthetized 8-month-old animals, a 2F microconductance pressure catheter (ARIA SPR-719; Millar Instruments Inc, Houston, Tex) was positioned via the right carotid artery in the left ventricle (LV) for continuous registration of LV pressure-volume loops. Volume signals were calibrated as previously described.16,17 Vascular resistance was calculated by the ratio of LV end-systolic pressure to stroke volume (mmHg/mL). Indexes of systolic and diastolic cardiac performance were obtained at steady state.

Van Gieson’s Fibrosis Staining

Paraffin sections were deparaffinized with xylene and hydrated with graded ethanol. The sections were subsequently placed in Weigert’s iron hematoxylin solution for 20 minutes, rinsed for 10 minutes, and placed in van Gieson’s stain for 5 minutes. Subsequently, the slides were placed in 95% ethanol and dehydrated with 100% ethanol. Grade of fibrosis was evaluated by a pathologist blinded to phenotype.

TUNEL Staining

The laboratory protocol was adapted18 and performed with Roche (Mannheim, Germany) reagents. Negative control was performed by omitting TdT. As a positive control, we used paraffin-embedded sections of 10% buffered formalin-fixed mammary glands (ApopTag Control Slides; Oncor, Gaithersburg, Md). After counterstaining with Harris’ hematoxylin, slides were dehydrated and mounted in Merckoglas (Merk, Darmstadt, Germany).

Western Blot Analysis and Immunodot Blotting

Protein was extracted from frozen tissue in cell extraction buffer containing 50 mmol/L Pipes/NaOH (pH 6.5), 2 mmol/L EDTA, 0.1% CHAPS, 5 mmol/L DTT, 20 μg leupeptin, 10 μg/mL pepstatin, 10 μg/mL aprotinin, and 1 mL/mL phenylmethylsulfonyl fluoride. Total protein (80 μg) was separated by electrophoresis on 4% to 12% polyacrylamide gel and blotted onto a polyvinylidene difluoride membrane. Western blots were performed using a standard protocol with a specific primary antibody to caspase 3 (Cell Signaling Technology, Beverly, Mass) at a dilution of 1:500 and with horse–radish peroxide–conjugated goat anti-rabbit secondary antibody (Jackson Immunoresearch Laboratories, West Grove, Pa) at a dilution of 1:10 000, Dako, Glostrup, Denmark). Caspase 3–specific signals were calibrated as previously described.16,17 Vascular resistance catheter (ARIA SPR-719; Millar Instruments Inc, Houston, Tex) was positioned via the right carotid artery in the left ventricle (LV) for continuous registration of LV pressure-volume loops. Volume signals were calibrated as previously described.16,17 Vascular resistance was calculated by the ratio of LV end-systolic pressure to stroke volume (mmHg/mL). Indexes of systolic and diastolic cardiac performance were obtained at steady state.

Mitochondrion Isolation

Mitochondria were isolated from the heart according to the method of Smith.20 Mitochondrial protein concentration was determined by BCA (bicinchoninic acid) test (Pierce, Bonn, Germany).

Analysis of Cytosolic Versus Mitochondrial Cytochrome C

Cytosolic fraction obtained from mitochondrion isolation was clarified by centrifugation at 16 400g for 1 hour at 4°C. Mitochondria were solubilized in 0.1 mol/L Na2SO4, 10 mmol/L Mops, pH 7.2, and 1% Triton X-100 and centrifuged for 10 minutes at 12 400g at 4°C. Then, 20 μg cytosolic and 7 μg mitochondrial protein were subjected to SDS gel electrophoresis, and Western blots were performed with anti–cytochrome c antibody in a dilution of 1:500 (Santa Cruz Biotechnology, Heidelberg, Germany). Cytochrome c–specific signals were normalized against loaded protein visualized by Coomassie Blue, and ratios between cytosolic to mitochondrial cytochrome c were calculated.

Electron Microscopy

After fixation in Karnovsky fixative followed by postfixation in 1% OsO4 solution and 0.1 mol/L phosphate buffer, samples were rinsed and dehydrated in ascending alcohol series as described by Shakibaie.21 They were embedded in Epon and cut on a Reichert-Jung Ultracut E (Heidelberg, Germany), followed by contrast treatment with 2% uranyl acetate/lead citrate. Ultrathin sections were prepared and evaluated with a transmission electron microscope (TE 10, Zeiss, Jena, Germany). The sarcomere length was determined by scoring 50 sarcomeres from 20 different microscopic fields of 3 hearts from each phenotype. The percentage of cells with morphological features of damaged mitochondria was determined by scoring 200 cells from 30 different microscopic fields of 3 hearts from each phenotype.

ANT Activity

Mitochondria were loaded with ATP, and ATP/ADP exchange was measured after addition of external ADP according to Passarella et al.22 The ATP Bioluminescence Assay CLSII (Roche, Mannheim, Germany) was used as the detection system.

Determination of Respiratory Chain Complex Activities

Complex I activity was measured by the oxidation of NADH by decylibiquinone at 340 nm; complex II activity, by the oxidation of succinate by decylubiquinone at 600 nm; complex III activity, by reduction of cytochrome c by decylubiquinol at 550 nm; and complex IV, by oxidation of dithionite-reduced cytochrome c at 550 nm as described previously.23 Mitochondrial ANT synthase activity was determined according to Yamada and Huzel24 using the ATP Bioluminescence Assay Kit CLSII from Roche (Mannheim, Germany) as the ATP detection system. The respiratory control ratio was determined as described by Pestosil et al23 with a Clark-type oxygen electrode (H. Saur, Reutlingen, Germany) and succinate as the respiratory substrate.

Measurement of ATP and ADP

Tissue probes were shock-frozen in liquid nitrogen and stored at −80°C. Tissue homogenization was performed in 10 vol% 0.4 mol/L perchloric acid in a glass Teflon potter and centrifuged at 16 000g at 4°C. Supernatant was neutralized with KHCO3 solution and centrifuged. The pellet was dissolved in 0.1 mol/L NaOH, and its protein content was determined with the BCA (bicinchoninic acid) assay from Pierce (Bonn, Germany). The ATP content of the supernatant was determined with the ATP Bioluminescence Assay Kit CLSII from Roche (Mannheim, Germany) according to manufacturer’s instructions. ADP was converted into ATP in a reaction of 50 mmol/L imidazole-HCl, pH 7.0, 2 mmol/L MgCl2, 75 mmol/L KCl, 3 mmol/L phosphoenol pyruvate, and 100 μg/mL pyruvate kinase. Reaction was stopped after 1 hour by adding 1 vol% 1 mol/L
NaOH and heating for 10 minutes at 60°C. Samples were analyzed for ATP content as described above.

Mitochondrial and Cytosolic Creatine Kinase Activity
Cytosolic creatine kinase activity was determined from high-spun cytosolic fraction obtained during mitochondria isolation. Cytosolic and mitochondrial creatine kinase activity was determined with the CK (NAC) Granutest 2.5 from Beckman Coulter (Fullerton, Calif). Cytosolic creatine kinase isoenzyme pattern was determined with the Paragon electrophoresis system from Beckman Coulter.

Statistical Analysis
Statistical analysis was performed by Student t test or the Mann-Whitney U test. Values are shown as mean±SEM of n independent experiments. Differences were considered significant at values of P<0.05.

The authors had full access to the data and take responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results
ANT1 Transgene Expression
We generated ANT rats (transgenic rats overexpressing rat ANT1). The construct was driven by the rat αMHC to ensure transgene expression in cardiomyocytes (Figure 1A). Transgene integration was proved by Southern blot, and the copy number of the transgene construct was ≈7 to 9 copies at a unique integration side (Figure 1B).

Although the highest expression of the transgene was detected in the ventricle and atrium, minor ectopic gene activity was visualized in brain but was not detectable in peripheral organs like kidney and lung (Figure 1C). The cardiac level of transgene RNA underwent ontogenetic regulation. Although the target RNA was identified at all investigated time points (Figure 1D), expression peaked in early adulthood. Importantly, transgenic RNA expression led to significantly higher ANT protein concentrations in myocardial mitochondria in both young (4-month-old) and older (8-month-old) rats (Figure 1E).

Cardiac Function in ANT Rats
ANT rats revealed no significant alterations in blood pressure (Figure 2A), heart rate (the Table), and ratio of heart to body weight (Figure 2B) compared with their age-matched WT controls. The LV function showed no significant difference in
systolic (LV end-systolic pressure and dp/dt max) or diastolic LV pressure parameters (LV end-diastolic pressure, dp/dt min, and time constant of isovolumic relaxation (τ)) between the 2 strains (the Table). In contrast, volume analysis revealed a significant elevation in LV end-systolic and LV end-diastolic volume in transgenic ANT1 rats, indicating an increase in LV chamber size. This was accompanied by an increase in stroke volume and cardiac output and a lower vascular resistance without changes in ejection fraction. The increase in chamber size was associated with a mild increase in sarcomere length, a marker significantly increased in ventricular dilation or structural alteration (Figure 2C). The ventricular wall thickness of ANT transgenic rats was not decreased, however, compared with their WT controls (data not shown).

**Hemodynamic Parameters of WT and Transgenic Rats**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT</th>
<th>ANT</th>
<th>REN</th>
<th>ANT/REN</th>
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<tr>
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<td>327±11</td>
<td>320±22</td>
<td>350±22</td>
<td>358±30</td>
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<tr>
<td>EF, %</td>
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<td>162±3.0†</td>
<td>158.3±0.3†</td>
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<td>LVEDP, mm Hg</td>
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<td>6.3±0.7</td>
<td>26.1±0.9†</td>
<td>15.1±2.1§</td>
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<tr>
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<td>527±46†</td>
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<tr>
<td>LVEDV, μL</td>
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<td>892±48§</td>
<td>439±41*</td>
<td>688±54**</td>
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<td>CO, mL/min</td>
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<td>116.8±9.4*</td>
<td>65.9±8.3*</td>
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<tr>
<td>SV, μL</td>
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<td>365±26*</td>
<td>187±27*</td>
<td>301±41§</td>
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<td>VR, mm Hg/μL</td>
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<td>0.27±0.03*</td>
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<td>τ, ms</td>
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<td>-3749±529</td>
</tr>
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</table>

EF indicates ejection fraction; LVSP, LV end-systolic pressure; LVEDP, LV end-diastolic pressure; LVEASP, LV end-systolic volume; LVEDV, LV end-diastolic volume; CO, cardiac output; SV, stroke volume; VR, vascular resistance; τ, time constant of isovolumic pressure relaxation; dp/dt max, maximal rate of left ventricular pressure rise; and dp/dt min, maximal rate of left ventricular pressure drop.

*P<0.05, †P<0.01, ‡P<0.001 vs WT, §P<0.05, †P<0.01 vs REN.

**ANT1 Overexpression Attenuates Myocardial Malfunction**

To investigate the impact of elevated ANT1 expression on cardiac pathophysiology, we crossed ANT animals with renin-overexpressing rats (REN rats), marked by fulminant hypertension leading to concentric cardiac hypertrophy and dysfunction, as well as high mortality. Although REN rats showed cardiac hypertrophy (Figure 2B) and a pronounced increase in sarcomere length (Figure 2C), systolic contractility (dp/dt max) was still preserved but diastolic function was impaired as shown by an increase in filling pressure (LV end-diastolic pressure), prolongation of LV relaxation (τ), and an upward and leftward shift of the pressure-volume loop (Figure 2D). The shift in the pressure-volume loop indicates a reduction in chamber volume (LV end-diastolic pressure). Consequently, cardiac output and stroke volume were significantly decreased.

Importantly, double-transgenic rats (ANT/REN rats) showed equal hypertension (Figure 2A) and elevation in LV systolic pressure as REN rats but a preserved relaxation (eg, τ) (the Table). Furthermore, afterload rose in REN rats but not in ANT/REN animals; thus, cardiac output and ejection fraction did not change compared with normotensive ANT rats despite a significant increase in LV end-diastolic pressure. Remarkably, this increase in LV end-diastolic pressure was much less pronounced in ANT/REN than in REN rats (4.1-fold for REN versus 2.4-fold for ANT/REN) and was not accompanied by an increase in heart weight (Figure 2B). Additionally, the pronounced increase in sarcomere length in REN rats also was significantly reduced in ANT/REN rats (Figure 2C). Taken together, LV contractile reserve and hemodynamic compensation mechanisms of ANT/REN rats were sufficient to preserve cardiac function (Figure 2D) and to prevent cardiac hypertrophy under hypertensive conditions, whereas REN rats showed evidence of cardiac end-organ damage.

**ANT1 Overexpression Decreases Cardiac Fibrosis**

Congruent to the hemodynamics and ratio of heart to body weight, ANT animals revealed no fibrotic changes within the myocardium or interstitial spaces. The REN group, however, showed marked interstitial fibrosis, characterized by dense collagenous tissue with multifocal replacement of cardiac myofibrils by connective tissue. These malformations were prevented in REN rats that coexpressed ANT1 (Figure 3A and 3B).

**ANT1 Overexpression Decreases Cardiac Apoptosis**

Because cardiac hypertension is associated with a high degree of apoptosis and stable ANT function has been shown to prevent apoptosis, apoptotic cells were quantified with TUNEL staining (Figure 4A and 4B). Whereas ANT1 overexpression alone did not alter the rate of apoptosis, REN rats were characterized by focal accumulation of positive-stained cells. The number of these cells was significantly decreased in ANT/REN rats. Furthermore, the ratio of cytosolic to mitochondrial cytochrome c was significantly increased in REN rats but less pronounced in ANT/REN rats (Figure 4C). In addition, the amount of caspase 3 was remarkably elevated.
by renin overexpression but normalized by transgenic ANT coexpression (Figure 4D).

Ultrastructural Analysis of Myocardial Morphology
To link our biochemical data of apoptosis to structural changes, we performed electron microscopy. WT and ANT rat hearts showed intact cardiomyocytes with well-developed numerous cardiomyofibers and organized intact mitochondria (Figure 5A and 5B). In contrast, REN rat hearts revealed disrupted contractile structures, nuclei with chromatid condensation, free swollen mitochondria, membrane blebbing, and cellular fragmentation, which are characteristic signs of programmed cell death (Figure 5C and 5D). However, double-transgenic rats revealed reduced pathological alteration in their myocardial ultrastructure with intact cardiomyocytes, well-organized cardiomyofibers, and significantly less damaged mitochondria (Figure 5E). The frequency of cells with focal mitochondrial changes was massively increased in REN rat hearts but significantly reduced in ANT/REN rats (Figure 5F).

ANT 1 Overexpression Is Accompanied by Increased Activity of Respiratory Chain Complexes
REN animals had a significantly reduced mitochondrial copy number that was preserved at normal level in ANT/REN rats (Figure 6A). To determine the mitochondrial function independently of the copy number, we analyzed the activities of the respiratory chain complexes in isolated mitochondria. In ANT and ANT/REN transgenic rats, the elevated ANT1 expression correlated with a significantly increased mitochondrial ATP/ADP transport (Figure 6B). The elevated ANT activity in ANT transgenic rats (Figure 6B) was paralleled by an increase in the activities of respiratory chain complexes II through IV (Figure 6C) and an elevation in the respiratory control ratio determined with succinate as the respiratory substrate (2.4±0.12 for WT versus 3.1±0.4 for ANT; P<0.05). REN rats showed normal ANT activity compared with WT rats but a deficiency in complex III and ATP synthase (complex V) activity (Figure 6C) that had been prevented in ANT/REN animals. Compared with WT rats, the amount of ATP (Figure 6D) and ADP (Figure 6E) was not significantly changed by ANT overexpression.

The mitochondrial creatine kinase activity (Figure 6F), functionally coupled to ANT, was not altered in ANT1-overexpressing animals. However, a reduced mitochondrial creatine kinase activity in REN rats has been restored by parallel overexpression of ANT1. The same effect has been found for the cytosolic creatine kinase activity (Figure 6G). In addition, the decrease in cytosolic creatine kinase is accompanied by a shift in the cytosolic creatine kinase isoform pattern from the MM to BB creatine kinase isoform (data not shown).

ANT1 Overexpression Improves the Survival Rate of Hypertensive Rats
To further characterize the benefit of the improved cardiac performance and tissue structure in ANT/REN rats compared with equally hypertensive REN rats, we analyzed the survival rate in all strains generating Kaplan-Meier curves. Until an age of 62 weeks, mortality was less pronounced in ANT/REN rats than in animals exclusively overexpressing renin (Figure 7A). However, although the cardiac phenotype was almost preserved in double transgenic rats, these animals still had a significantly lower survival rate than WT or ANT animals.

Besides cardiac pathology, hypertension also mediates damage in other end organs such as kidneys (renal failure) and brain (stroke). We therefore hypothesized that the lack of ANT1 overexpression in the kidneys may still promote renal pathology and consequently lead to the increased mortality in ANT/REN rats compared with WT and ANT animals. The REN and ANT/REN group evidenced marked interstitial fibrosis (Figure 7B, middle) but
showed differences in the degree of glomerular damage (Figure 7B, right). The REN group revealed prominent multifocal thickening of Bowmann’s capsule with obliteration of Bowmann’s space (Figure 7B, left and right). Moreover, the REN group frequently showed glomerular sclerosis and glomerular atrophy. Compared with REN, the ANT/REN group revealed slightly improved glomerular structure and occasionally still mild glomerular sclerosis. Thus, morphological changes such as glomerular collapse, thickening of basement membranes, and cortical and interstitial medullary fibrosis were most prominent in the REN group and less pronounced in the ANT/REN kidneys.

**Discussion**

Cardiac hypertrophy is linked to disturbed mitochondrial function associated with restricted oxidative phosphorylation, β-oxidation, and impaired mitochondrial and cytosolic energy transfer. These restrictions are in turn linked to intensified apoptosis, necrosis, and mitochondrial damage. ANT is a multifunctional protein that is involved in several of these processes because it facilitates mitochondrial ATP/ADP transfer and contributes to the regulation of apoptosis. We were now able to show that increasing ANT transport capacity as a result of transgenic ANT1 expression positively affects oxidative phosphorylation and apoptosis and is an effective approach to maintain mitochondrial and thus cardiac function in hypertension-induced heart disease.

ANT rats revealed a cardiac phenotype characterized by an increase in cardiac output resulting from an increase in chamber size without changes in ejection fraction. Because this LV remodeling was associated with mild fiber stretch but not with an increase in fibrosis and apoptosis, a cardiac maladaptation appears unlikely.

Systemic hypertension such as that found in REN animals is known to cause an increase in cardiac mass, inducing early diastolic dysfunction indicated by increased filling pressures. This impairment was significantly improved by parallel ANT1 overexpression as shown by restored LV diastolic relaxation (τ, LV end-diastolic pressure) and compliance under hypertensive conditions. Importantly, these ANT/REN rats did not develop significant LV hypertrophy or fibrosis, indicating that the cardiac reserve resulting from molecular changes by ANT overexpression was able to cope with high blood pressure without compensating with cardiac hypertrophy. The slight left-
The backward shift of the pressure-volume loop had not reached a pathological impact. Consequently, an overexpression of ANT1 protects REN animals against hypertension-induced cardiac remodeling. Further experiments have to prove this finding in heart failure models of other origins, however. Furthermore, it remains unclear how long an increased ANT expression is able to compensate for a renin-induced hypertensive stimulus persistently stressing the heart.

Currently, elevated ANT1 expression is discussed as being part of a cardiomyocyte-protective program because the expression of genes encoding complexes in oxidative phosphorylation such as cytochrome oxidase, cytochrome c, and ANT is increased after ischemic or hyperthermic preconditioning.10,34,35 Preconditioned mitochondria demonstrated a remarkable preservation of oxidative phosphorylation after such short cardiac stress impulses. Cardiac overexpression of Bcl-2, an antiapoptotic protein that also

Figure 5. Representative electron micrographs of WT (A) and ANT transgenic hearts (B) revealed well-developed numerous cardiomyofibers and organized intact mitochondria (white arrows). Hearts of REN rats (C, D) showed clusters of degenerated myofibers, free and degenerative, swollen mitochondria (black arrows), and cardiomyocytes with manifested typical features of apoptosis, eg, membrane blebbing, nuclear changes (*) with peripheral segregation, and aggregation of chromatin into dense areas (D). ANT/REN hearts (E) represent an intact myocardial structure and clear reduction in pathological changes described for REN rats. Magnification ×20 000. (F) Percentages of cells with mitochondrial changes per 200 investigated cells. ***P<0.001 vs WT, ###P<0.001 vs REN.
increases the ADP/ATP transport activity of ANT, has been shown to support this protective effect. These findings are in agreement with our results that ANT1 overexpression is linked to increased activity of oxidative phosphorylation complexes in single ANT and ANT/REN rats. Notably, the amount of cellular ATP and ADP was not significantly altered in ANT transgenic animals; thus, the beneficial effect appears to be related to a faster energy transfer and not to an increase in total amount of ATP molecules. Furthermore, ANT1 overexpression also compensates for the lowered complex III, ATP synthase, and creatine kinase activity found in REN heart mitochondria. Although it did not influence ATP synthase and mitochondrial creatine kinase activity under basic conditions, it reversed their malfunction in REN rats. This indicates that the normalization of both enzymes is secondarily rather than directly affected by ANT overexpression. The generalized improvement also is transferred to the cytosolic creatine kinase system shown by normalized cytosolic creatine kinase activity and isoform pattern reshift. Taken together, the ANT overexpression leads directly and indirectly to restored energy production and transport in REN rats. Thus, consequent improvement in mitochondrial function is a prerequisite for the general cardiac improvement observed in ANT/REN rats.

The positive effect of such an improved energy supply may be, among others, the inhibition of mitochondrion-related apoptosis seen in ANT-overexpressing REN rats. Malfuction of oxidative phosphorylation observed in REN animals induces the opening of the mitochondrial permeability transition pore, triggering apoptosis. ANT is a component of the mitochondrial permeability transition pore and has a regulating function on the pore. Mitochondrial permeability transition pore opening leads to mitochondrial swelling and release of caspase-activating factors such as cytochrome c, resulting in the DNA degradation and cell damage found in the REN rats. ANT1 overexpression in REN rats, however, is accompa-

Figure 6. Transgenic ANT1 expression is accompanied by increased respiratory chain complex activities. A, Ratio of mitochondrial DNA (CytB gene) to genomic DNA (18s rRNA gene) determined by real-time polymerase chain reaction. B, ADP/ATP exchange was measured in isolated, ATP-loaded mitochondria. C, The activity of each respiratory chain complex (CI-CV) was measured from isolated cardiac mitochondria of WT (white), ANT (black), REN (gray), and ANT/REN (shaded bar) rats. Values were normalized to the WT data and expressed as percentages. Total ATP (D) and ADP (E) was measured by luminescence assays. Mitochondrial (mt; F) and cytosolic (cyt; G) creatine kinase activity was measured by optical tests. N=6 per strain. *P<0.05, **P<0.01, §P=0.056 vs WT; #P<0.05, ##P<0.01, ###P<0.001 vs REN.
nied by a stabilization of the respiratory chain reaction that significantly contributes to the inhibition of pore opening shown by prevented mitochondrial cytochrome c release, normal caspase 3 expression, preserved mitochondrial structure and mitochondrion number, and a smaller number of apoptotic nuclei. In fact, myocardial ANT1 overexpression increases the tightness of the inner mitochondrial membrane for ions indicated by an increased respiratory control ratio, a parameter for the mitochondrial membrane permeability and effectiveness of energetic coupling. Following this reasoning, reduced cell death is reflected by preserved cell structure with a well-organized contractile apparatus, reduced rate of tissue replacement, and fibrosis in the ANT/REN rats. This in turn further improves cardiac function and enhances survival of these animals. Remarkably, the apoptosis-inhibiting and tissue-preserving effect of elevated ANT1 in pressure-stressed cardiomyocytes contradicts findings of an apoptosis-inducing impact by ANT1 and ANT3 overexpression in cultured HeLa cells. In contrast to cardiomyocytes, HeLa cells express mainly ANT2 but little ANT1 and ANT3 (unpublished data). Because apoptosis was not observable in ANT transgenic animals, apoptosis-influencing effects of increased ANT1 expression appears to be cell type and ANT isoform pattern dependent.

Interestingly, the survival rate of ANT/REN rats is not completely restored, although cardiac function is almost preserved. However, because systemic hypertension leads

Figure 7. Survival rate and renal histology. A, Kaplan-Meier curves illustrating survival in the 4 investigated strains over 62 weeks. (WT, n=20 rats; ANT, n=27 rats; REN, n=57 rats, ANT/REN, n=45 rats). B, Histological images of renal sections stained by hematoxylin and eosin (left; magnification ×10) and van Gieson (middle, right; magnification, ×20) stain. *P<0.05, ***P<0.01 vs WT; #P<0.05 vs REN.
to end-organ damage in a variety of other organs besides the heart, cardiac ANT1 overexpression cannot protect all organs from a hypertension-induced pathology. This was strongly confirmed by moderated but still severe kidney alterations in our ANT/REN rats, indicating that the high mortality in REN rats is mediated only partly by cardiac failure but also may be caused by renal insufficiency.

Our data clearly show that an accelerated ATP/ADP transportation across the mitochondrial membrane improves mitochondrial structure and function and thus ameliorates tissue integrity and cardiac performance under pathological conditions. The prevention of cardiac damage in ANT1-overexpressing REN rats illustrates the promising potency of ANT1 stimulation as a new tool in the therapy of heart disease.

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Disclosures
None.

References


37. Ly JD, Grubb DR, Lawen A. The mitochondrial membrane potential (\(\Delta \psi_m\)) in apoptosis; an update. *Apoptosis*. 2003;8:115–128.


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**CLINICAL PERSPECTIVE**

Strong evidence recently arose indicating that mitochondria malfunction is a linchpin in the induction and facilitation of cardiac failure disturbing energy metabolism and stimulating apoptosis. The adenine nucleotide translocase (ANT) facilitates the exchange of ADP and ATP across the inner mitochondrial membrane and thus plays a significant role in the regulation of cardiac energy metabolism. To verify the hypothesis that accelerated ATP/ADP transport can prevent or delay cardiac pathology, we generated transgenic rats specifically overexpressing ANT1 in the heart and crossed them with renin-overexpressing rats developing fulminant hypertension combined with cardiac hypertrophy and malfunction. Cardiac-specific ANT1 overexpression resulted in a higher ATP/ADP transportation and elevated activities of the respiratory chain complexes. Hypertension-induced cardiac hypertrophy in the renin-overexpressing rats was prevented by parallel ANT1 overexpression, and left ventricular function remarkably improved. ANT1 overexpression led to reduced fibrosis and improved cardiac tissue architecture. Consequently, the survival rate of ANT/renin–overexpressing rats was enhanced. Further investigations into the cardioprotective mechanism of ANT1 overexpression revealed improved mitochondrial structure and function and significantly reduced apoptosis in double-transgenic rats. Thus, the improvement of mitochondrial function may be a basic principle for new strategies in treating heart disease.
Accelerated Mitochondrial Adenosine Diphosphate/Adenosine Triphosphate Transport Improves Hypertension-Induced Heart Disease

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