Chronic ventricular myocyte-specific overexpression of angiotensin II type 2 receptor results in intrinsic myocyte contractile dysfunction

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Nakayama, Masaharu, Xinhua Yan, Robert L. Price, Thomas K. Borg, Kenta Ito, Atsushi Sanbe, Jeffrey Robbins, and Beverly H. Lorell. Chronic ventricular myocyte-specific overexpression of angiotensin II type 2 receptor results in intrinsic myocyte contractile dysfunction. Am J Physiol Heart Circ Physiol 288: H317–H327, 2005. First published September 16, 2004; doi:10.1152/ajpheart.00957.2003.—ANG II type 2 receptor (AT2) is upregulated in failing hearts, but its effect on myocyte contractile function is not known. We measured fractional cell shortening and intracellular Ca2+ concentration transients in left ventricular myocytes derived from transgenic mice in which ventricle-specific expression of AT2 was driven by the myosin light chain 2v promoter. Confocal microscopy studies confirmed upregulation of AT2 in the ventricular myocytes and partial colocalization of AT2 with AT1. Three components of contractile performance were studied. First, baseline measurements (0.5 Hz, 1.5 mmol/l extracellular Ca2+) at 25°C revealed Ca2+-dependent contractile dysfunction in myocytes from AT2 transgenic mice. Comparison of two transgenic lines suggested a dose-dependent relationship between magnitude of contractile dysfunction and level of AT2 expression. Second, activity of the Na+/H+ exchanger, a dominant transporter that regulates beat-to-beat intracellular pH, was impaired in the transgenic myocytes. Third, the inotropic response to β-adrenergic versus ANG II stimulation differed. Both lines showed impaired contractile response to β-adrenergic stimulation. ANG II elicited an increase in contractility and intracellular Ca2+ in wild-type myocytes but caused a negative inotropic effect in myocytes from AT2 transgenic mice. In contrast with β-adrenergic response, the depressed response to ANG II was related to level of AT2 overexpression. The depressed response to ANG II was also present in myocytes from young transgenic mice before development of heart failure. Thus chronic overexpression of AT2 has the potential to cause Ca2+- and pH-dependent contractile dysfunction in ventricular myocytes, as well as loss of the inotropic response to ANG II.

The cardiovascular effects of ANG II on vasomotor tone, growth, and contractility are mediated via the ANG II type 1 receptor (AT1), whereas the ANG II type 2 receptor (AT2) is postulated to promote apoptosis and suppress growth (13, 25). The expression level of AT2 in adult heart is nearly undetectable (11, 32), whereas the proportion of AT2 to AT1 is variably increased in ventricles of failing human hearts (4, 12, 33) and hypertrophied rat hearts (23). These observations suggest that the chronic upregulation of AT2 in ventricles may play a role in the progression of heart failure.

Studies using genetic manipulation of AT2 have provided conflicting results. Akishita et al. (3) showed that pressure overload caused similar cardiac hypertrophy in wild-type (WT) and AT2-null mice. In contrast, two studies demonstrated that neither pressure overload nor ANG II induced left ventricular (LV) hypertrophy in AT2-null mice, suggesting that AT2 is essential for cardiac hypertrophy (14, 29). Masaki et al. (24) reported that mice with AT2 expression driven by the α-myosin heavy chain promoter showed no apparent change in cardiac morphology or LV function. However, they observed a confounding effect of depressed chronotropic response to ANG II, possibly due to AT2 overexpression in atria, which may chronically modulate workload.

To more precisely evaluate the function of AT2 in ventricles, we produced transgenic (TG) mice in which the myosin light chain (MLC)2v promoter was used to drive TG expression of AT2 specifically in the ventricular cardiomyocytes. We recently reported (34) that myocyte-specific AT2 overexpression is associated with the in vivo phenotype of heart failure associated with cardiac chamber dilatation, remodeling, and apoptosis. No prior study has examined the effect of AT2 expression on myocyte contractile function. The present study tested the hypothesis that chronic AT2 expression modifies contractility and intracellular Ca2+ concentration ([Ca2+]i) transients in isolated LV myocytes from AT2 TG mice compared with WT mice. We compared two lines of heterozygous TG mice to evaluate the effects of different expression levels of AT2 and examined three components of integrated contractile function that contribute to contractile reserve in vivo. These include 1) the augmentation of contractility at faster depolarization rates, 2) the capacity to increase contractility in response to the inotropic agonists ANG II and isoproterenol, and 3) the rapid correction of intracellular acidification.

MATERIALS AND METHODS

Animal preparation. TG mice with ventricle-specific overexpression of AT2 were generated as described previously (34). Briefly, the AT2-expressing transgene driven by the MLC2v promoter was microinjected into the pronuclei of single cell fertilized mice to generate TG mice (FVB/n strain). High (AT2HG; copy number = 18)- and low (AT2LG; copy number = 9)-expressing lines were selected to
determine the dose-response relationships. Expression of the AT₂ transgene in both ventricles with negligible atrial expression was confirmed by RT-PCR using AT₂-specific primers (20). Expression of AT₁ was similar between the WT and TG lines. The steady-state AT₂ mRNA level in the AT₁<sup>WT</sup> TG line was 4.04-fold higher than that in the AT₁<sup>WT</sup> line, as measured by quantitative real-time RT-PCR. Western blotting analysis showed that the LV AT₂ expression level in AT₁<sup>WT</sup> TG mice was 4.27-fold higher than that in AT₁<sup>WT</sup> TG mice, whereas AT₂ protein expression in LV of WT mice was virtually nil (34). In the present study, mice were studied between 18 and 22 wk of age, when the phenotype of dilated cardiomyopathy with overt heart failure became evident in the AT₁<sup>WT</sup> TG mice, and between 4 and 5 wk of age, when heart failure was not yet evident in AT₁<sup>WT</sup> TG mice. Gender differences in myocardial contractility were not observed in preliminary studies, and subsequently data were collected from both sexes.

**Confocal microscopy analysis of AT₁ and AT₂.** LV myocytes from WT or TG mice were isolated by a collagenase perfusion method (15, 16) and fixed overnight in 4% paraformaldehyde (pH 7.2). Myocytes were stained for AT₁ (Research Diagnostics; 6.7 μg/ml), AT₂ (Santa Cruz Biotechnology; 0.4 μg/ml) and F-actin (1:50 rhodamine phallolidin; Molecular Probes). AT₂ and AT₁ were imaged with Cy2 and Cy5, respectively (Jackson ImmunoResearch). Controls included the use of normal sera and the replacement of primary antibodies with PBS while staining. All controls were negative. Myocytes were mounted in a 1:3 solution of PBS-glycerine with 1,4-diazabicyclo[2.2.2]octane (DABCO) to reduce photobleaching. Myocytes from a minimum of five fields at a magnification of ×10 were examined from each group. The staining patterns and intensity were similar for each field of a given experimental condition. Representative images were collected on a Bio-Rad MRC 1024ES confocal microscope equipped with a Kr/Ar laser. For the collection of the AT₂ images from WT and AT₁<sup>WT</sup> TG myocytes, all operating parameters including laser intensity, gain, and offset were kept consistent. For the collection of the AT₁ images from AT₁<sup>WT</sup> TG myocytes, where it was necessary to reduce the gain in the Cy2 channel to collect acceptable digital images showing colocalization of AT₂ with AT₁, we also present data where the color of AT₁ is changed to red, such that yellow is the merged signal of AT₁ (red) and AT₂ (green).

**Myocyte function and [Ca<sup>2+</sup>]<sup>i</sup> measurements.** Isolated myocytes were superfused with control solution containing (in mmol/l) 137 NaCl, 4.0 HEPES, 0.5 MgSO<sub>4</sub>, 3.7 KCl, 5.6 glucose, and 0.5 probenecid with a final pH of 7.4 at 25°C. Under baseline conditions, myocytes were paced with field stimulation at 0.5 Hz at 25°C. To study contractile reserve in response to an increase in pacing frequency, the pacing rate was increased from 1 to 5 Hz, in increments of 1 Hz, with constant extracellular Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>o</sub>) at pH 7.4 (36°C). Excitation was performed at 540 nm, and fluorescence emission was collected simultaneously at 580 and 640 nm. The pH for each cell was calibrated as described previously (18, 30). The rate of acid efflux (J<sub>H</sub>) was used as the indicator of sarcolemmal Na<sup>+</sup>/H<sup>+</sup> exchanger (NHE) activity according to the formula: J<sub>H</sub> = β × ΔpH/Δt (26), where β is intrinsic buffering power. J<sub>H</sub> values were determined at intervals of 0.5 min during recovery from intracellular acidosis after a rapid pulse (2 min) exposure to 10 mmol/l NH₄Cl. Buffering power β<sub>H</sub> was separately estimated by using stepped reduction in trimethylamine (TMA) hydrochloride (Sigma) to cause stepwise changes in pH as previously described in isolated myocytes (18): β<sub>H</sub> = Δ[TMAH<sup>+</sup>]/ΔpH<sub>H</sub>, where [TMAH<sup>+</sup>]<sub>i</sub> is the intracellular concentration of TMA ions, which is calculated as [TMAH<sup>+</sup>]<sub>i</sub> = [TMA]<sub>i</sub>[H<sup>+</sup>]<sub>i</sub> + 10<sup>pH<sub>H</sub>-pK<sub>H</sub></sup>, where [TMA]<sub>i</sub> is the total extracellular concentration of TMA and pK<sub>H</sub> is the dissociation constant, taken as 9.80 (18). The relationship between β<sub>H</sub> and pH<sub>H</sub>, where pH<sub>H</sub> was taken at the midpoint of each pH step, was plotted as the line fit by least-squares linear regression for the WT and TG myocytes. In this protocol, there were no differences among the groups in values of β<sub>H</sub> used for the calculation of J<sub>H</sub> [β<sub>H</sub>, (mmol/pH unit)] = 191.7 - 24.8 pH<sub>H</sub> (n = 40 measurements from 10 WT myocytes), 171.9 - 21.1 pH<sub>H</sub> (n = 26 measurements from 7 AT<sub>low</sub> TG myocytes), and 183.2 - 22.3 pH<sub>H</sub> (n = 28 measurements from 8 AT<sub>high</sub> TG myocytes).

**Protein levels in LV myocytes and tissue.** Western blotting was performed to assess LV protein levels of sarco(endo)plasmic reticulum Ca<sup>2+</sup>-ATPase (SERCA2) and phospholamban (PLB) with specific antibodies (Affinity Bioreagents) (15, 16). The optical density of the immunoblots on films was measured with NIH Image software. Measurements were normalized with GAPDH (n = 8–10 hearts/group). Levels of NHE-1, endothelial nitric oxide synthase (eNOS), and inducible nitric oxide synthase (iNOS) proteins were also measured with anti-NHE-1 antibody (Chemicon International), anti-eNOS antibody (BD Biosciences), and anti-iNOS antibody (BD Biosciences) in WT (n = 5), AT₁<sup>WT</sup> TG (n = 5), and AT₁<sup>WT</sup> TG (n = 5) mice.

**Statistical analysis.** Values are expressed as means ± SE. Comparison among groups was analyzed by ANOVA followed by post hoc testing using the Tukey test. Two-way ANOVA with repeated measurements was used for statistical analysis of the contractile reserve studies, which assessed sequential changes in pacing rate, isoproterenol concentration, and ANG II concentration, respectively. Statistical significance was accepted at the level of P < 0.05.
Fig. 1. A: confocal microscopy study of myocytes isolated from wild-type (WT) (top), low ANG II type 2 receptor (AT2)-expressing (AT2<sub>low</sub>) transgenic (TG) (middle), and high AT2-expressing (AT2<sub>high</sub>) TG (bottom) mice. **Left**, myocyte morphology shown by staining for F-actin with rhodamine phalloidin. **Center**, staining for AT2, which was very weak in WT and the highest in AT2<sub>high</sub> TG mice. **Right**, corresponding staining for ANG II type 1 receptor (AT1) in each group. B: double staining for AT2 (green) with AT1 (red) in AT2<sub>low</sub> TG myocytes shows colocalization (yellow) of AT2 with AT1. This suggests that at least a subpopulation of AT2 receptors can, in fact, colocalize with AT1 receptors in isolated myocytes. Scale bar, 50 μm.
RESULTS

Confocal imaging of cardiomyocyte AT₁ and AT₂. To ensure that AT₂ are present in collagenase-dissociated myocytes from TG mice used in these experiments, confocal microscopic analyses were performed (Fig. 1A). Staining intensity for AT₂ was extremely low in WT myocytes, consistent with prior reports in normal postnatal myocytes (11, 32, 34). As expected, staining intensity for AT₂ was highest in AT₂⁰⁰⁰⁰ TG myocytes whereas AT₂⁰⁰⁰⁰ TG myocytes showed a staining intensity that was obviously less than that of AT₂⁰⁰⁰⁰ TG myocytes but still readily detectable. In contrast, AT₁ expression appeared almost identical among all three groups of cardiomyocytes. This observation corroborates the prior finding that expression levels of AT₁ are similar in WT and TG lines of the model (34).

Recently, AbdAlla et al. (1) reported that AT₂ formed heterodimers with AT₁ in multiple tissues. Figure 1B shows a merged image of AT₁ (red) and AT₂ (green), indicating that a subpopulation of these receptors do colocalize (yellow) in isolated cardiomyocytes.

Characteristics of hearts and myocytes from AT₂ TG mice.

Both LV weight and the LV weight-to-body weight ratio were significantly increased in AT₂⁰⁰⁰⁰ TG compared with AT₂⁰⁰⁰⁰ TG and WT mice, whereas body weight was identical in all groups from 18 to 22 wk of age (Table 1). Table 2 shows the baseline characteristics of cell morphology contraction and [Ca²⁺]i transients in myocytes from WT and TG mice from 18 to 22 wk of age. Myocyte cell area and diastolic cell length were greater in AT₂¹⁰⁰⁰⁰ TG compared with AT₂⁰⁰⁰⁰ TG and WT mice. Fractional cell shortening was severely depressed in AT₂¹⁰⁰⁰⁰ TG myocytes, and mildly depressed in AT₂⁰⁰⁰⁰ TG myocytes compared with WT myocytes (4.0 ± 0.3% and 5.2 ± 0.4% vs. 6.9 ± 0.5%, P < 0.01 and P < 0.05, respectively). Peak systolic [Ca²⁺]i and amplitude of [Ca²⁺]i were also decreased in AT₂¹⁰⁰⁰⁰ TG compared with WT myocytes. The Ca²⁺ transients in AT₂¹⁰⁰⁰⁰ TG myocytes were not different from those in WT myocytes, and end-diastolic [Ca²⁺]i was unchanged. Time to peak cell shortening and time to peak [Ca²⁺]i were also identical, but time to 50% cell relengthening and time to 50% decline in [Ca²⁺]i were prolonged in AT₂¹⁰⁰⁰⁰ TG mice compared with both AT₂⁰⁰⁰⁰ TG and WT animals. We also determined whether contractile dysfunction characteristic of heart failure was present in myocytes of young TG mice. There were no differences in LV weight or LV weight-to-body weight ratio (Table 1) or parameters of myocyte contractile function (Table 2) among the groups of mice between 4 and 5 wk of age.

Frequency-dependent contractile reserve. The augmentation of contractile function at rapid rates depends on both sarcoplasmic reticulum (SR)-dependent augmentation of Ca²⁺ stores and coordinated Ca²⁺-induced Ca²⁺ release. To assess rate-dependent contraction, we increased the stimulation rate from 1 to 5 Hz in 1-Hz steps in the presence of 1.5 mmol/l [Ca²⁺]o. In WT myocytes, fractional cell shortening increased in response to pacing frequency, associated with the augmentation of peak systolic [Ca²⁺]i, as shown in Figure 2, A and B. In contrast, fractional cell shortening and peak systolic [Ca²⁺]i were severely depressed at all pacing rates in AT₂⁰⁰⁰⁰ TG myocytes. The response of the myocytes from AT₂¹⁰⁰⁰⁰ TG mice was intermediate between WT and AT₂¹⁰⁰⁰⁰ TG mice. Diastolic [Ca²⁺]i in response to fast pacing frequency was not different among the groups at all pacing rates.

Calcium regulatory proteins. To elucidate potential underlying molecular mechanisms, we examined the protein levels of Ca²⁺-regulated PLB and SERCA2. PLB was increased in AT₂¹⁰⁰⁰⁰ TG compared with WT myocytes, whereas SERCA2 was similar among these groups. The ratio of SERCA2 to PLB was significantly depressed in AT₂¹⁰⁰⁰⁰ TG but not in AT₂⁰⁰⁰⁰ TG myocytes compared with WT myocytes (Fig. 3).

Isoproterenol-dependent contractile reserve. We next studied the contractile response to inotropic agonists that modulate PRKGC1 and PDE4B expression.

Table 1. Animal characteristics and LV weight

<table>
<thead>
<tr>
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<th>WT</th>
<th>AT₂⁰⁰⁰⁰ TG</th>
<th>AT₂¹⁰⁰⁰⁰ TG</th>
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<tbody>
<tr>
<td>n</td>
<td>31</td>
<td>26</td>
<td>34</td>
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<tr>
<td>Age, wk</td>
<td></td>
<td></td>
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<tr>
<td>Body wt, g</td>
<td>30.4 ± 0.3</td>
<td>29.7 ± 0.4</td>
<td>30.2 ± 0.3</td>
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<tr>
<td>LV wt, mg</td>
<td>150 ± 4.2</td>
<td>152 ± 7.7</td>
<td>171 ± 6.2</td>
</tr>
<tr>
<td>LV wt/body wt, mg/g</td>
<td>4.9 ± 0.2</td>
<td>5.1 ± 0.2</td>
<td>5.7 ± 0.2</td>
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<thead>
<tr>
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<th>4–5 wk of Age</th>
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<tbody>
<tr>
<td>n</td>
<td>12</td>
</tr>
<tr>
<td>Age, wk</td>
<td>4.6 ± 0.1</td>
</tr>
<tr>
<td>Body wt, g</td>
<td>20.0 ± 0.6</td>
</tr>
<tr>
<td>LV wt, mg</td>
<td>107 ± 3</td>
</tr>
<tr>
<td>LV wt/body wt, mg/g</td>
<td>5.4 ± 0.2</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE for n animals. WT, wild-type; AT₂, ANG II type 2 receptor; AT₂⁰⁰⁰⁰, low AT₂ expressing; AT₂¹⁰⁰⁰⁰, high AT₂ expressing; TG, transgenic; *P < 0.05 compared with WT; †P < 0.01 compared with AT₂⁰⁰⁰⁰ TG; ‡P < 0.01 compared with AT₂⁰⁰⁰⁰ TG.

Table 2. Baseline characteristics of myocyte contractile and [Ca²⁺]i transients

<table>
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<th>18–22 wk of Age</th>
<th>4–5 wk of Age</th>
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<tbody>
<tr>
<td>Myocyte area, μm²</td>
<td>2.553 ± 0.130</td>
<td>2.644 ± 0.122</td>
</tr>
<tr>
<td>Fractional cell shortening, %</td>
<td>6.9 ± 0.5</td>
<td>5.2 ± 0.4 *</td>
</tr>
<tr>
<td>Time to peak shortening, ms</td>
<td>125 ± 4</td>
<td>127 ± 3</td>
</tr>
<tr>
<td>Time to 50% shortening, ms</td>
<td>65 ± 5</td>
<td>68 ± 6</td>
</tr>
<tr>
<td>Amplitude of [Ca²⁺]i, nmol/l</td>
<td>395 ± 21</td>
<td>398 ± 10</td>
</tr>
<tr>
<td>Peak systolic [Ca²⁺]i, nmol/l</td>
<td>65 ± 3</td>
<td>69 ± 3</td>
</tr>
<tr>
<td>Time to peak [Ca²⁺]i, ms</td>
<td>45 ± 1</td>
<td>46 ± 2</td>
</tr>
<tr>
<td>Time to 50% decline in [Ca²⁺]i, ms</td>
<td>122 ± 3</td>
<td>123 ± 4</td>
</tr>
<tr>
<td>LV wt, mg</td>
<td>107 ± 3</td>
<td>116 ± 4</td>
</tr>
<tr>
<td>LV wt/body wt, mg/g</td>
<td>5.4 ± 0.2</td>
<td>5.4 ± 0.2</td>
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Values are expressed as means ± SE for n experiments 18–22 wk: n = 23 from 4 hearts (WT), 22 from 4 hearts (AT₂⁰⁰⁰⁰ TG), and 41 from 5 hearts (AT₂¹⁰⁰⁰⁰ TG); 4–5 wk: n = 26 from 3 hearts (WT), 22 from 3 hearts (AT₂⁰⁰⁰⁰ TG), and 21 from 3 hearts (AT₂¹⁰⁰⁰⁰ TG). Measurements were obtained at a pacing frequency of 0.5 Hz. *P < 0.05 compared with WT; †P < 0.01 compared with AT₂⁰⁰⁰⁰ TG; ‡P < 0.01 compared with AT₂¹⁰⁰⁰⁰ TG.
bead-to-beat contraction in normal myocytes. To examine the effect of β-adrenergic stimulation on inotropic response, we studied the response to isoproterenol (1–1,000 nmol/l). Figure 4 shows the dose-dependent curves of cell shortening and peak systolic [Ca²⁺], to isoproterenol. WT myocytes exhibited a progressive increase in fractional cell shortening and peak systolic [Ca²⁺], in a dose-dependent manner. In contrast, the response to isoproterenol was reduced in myocytes from both lines of AT₂ TG mice compared with WT mice. The response to isoproterenol did not distinguish myocytes from the lines with low versus high levels of AT₂ expression.

**Contractile function in response to ANG II.** To examine the response to ANG II, myocytes were stimulated with incremental changes in ANG II concentration (1–100 nmol/l). Figure 5, A and B, shows that ANG II elicited a positive inotropic effect in WT myocytes, related to an increase in peak systolic [Ca²⁺]. In contrast, in AT₂ hiTG myocytes, ANG II promoted a negative inotropic effect (P < 0.001 in fractional cell shortening and P < 0.001 in peak systolic [Ca²⁺]). The response of AT₂ loTG myocytes was intermediate. The effects of losartan, an AT₁ antagonist, and PD-123319, an AT₂ antagonist, were then studied in the presence of ANG II in WT and AT₂ hiTG myocytes. As expected, the positive inotropic effects of ANG II on contraction and peak systolic [Ca²⁺] in WT myocytes were inhibited by cotreatment with losartan (1 μmol/l) but not PD-123319 (100 nmol/l) (Fig. 5C). A novel and unexpected observation was that the marked negative inotropic effects of ANG II in AT₂ hiTG myocytes were also inhibited by cotreatment with losartan (1 μmol/l) but not PD-123319 (100 nmol/l) (Fig. 5D). We also observed that the AT₂ agonist CGP-42112A did not alter the contractility in either AT₂ TG or WT myocytes under baseline conditions in the absence of ANG II (data not shown).

To determine whether this negative inotropic response to ANG II was related to AT₂ expression or was a secondary consequence of late development of heart failure, this was also investigated in WT and TG mice between 4 and 5 wk of age, when heart failure was not yet developed in AT₂ hiTG myocytes. There were no differences in the baseline characteristics of isolated myocytes among the groups (Table 2). In WT myocytes, ANG II induced a positive inotropic effect, which was abolished by losartan (1 μmol/l) but not PD-123319 (100 nmol/l) (Fig. 6, A–C). The inotropic response to ANG II in AT₂ loTG myocytes was blunted (Fig. 6, A and B). ANG II caused a negative inotropic effect in AT₂ hiTG myocytes in a dose-dependent manner. Losartan, but not PD-123319, diminished the negative response (Fig. 6, A, B, and D).

**pH regulation in response to intracellular acidification.** The capacity to correct intracellular acidosis is an important additional factor that modulates normal beat-to-beat contractile function. Thus we studied pH, and NHE activity in myocytes from WT mice and both lines of AT₂ TG mice. Basal pH values among the groups were similar (Table 3). To assess the recruitment of NHE activation in response to acute intracellular acidification, myocytes were exposed to a rapid pulse and washout of 10 mmol/l NH₄Cl in bicarbonate-free HEPES solution (control solution). The NH₄Cl pulse caused the expected response of initial intracellular alkalosis, secondary intracellular acidosis, and recovery from acidosis to baseline pH, which was abolished by the NHE inhibitor 5-(N-ethyl-N-isopropyl)amiloride (10 μmol/l; data not shown). Figure 7A

![Figure 2](http://www.ajpheart.org/)

Fig. 2. Frequency-dependent contractile reserve in left ventricular (LV) myocytes from WT, AT₂ hiTG, and AT₂ loTG mice. A: relationship between pacing frequency and fractional cell shortening. B: relationship between pacing frequency and diastolic cell length. There were no significant differences in change of diastolic cell lengths among the groups. All hearts were obtained from mice between 18 and 22 wk of age; n = 20 experiments from 4 hearts (WT), 18 from 4 hearts (AT₂ hiTG), and 22 from 5 hearts (AT₂ loTG). Statistical analysis was performed by ANOVA with repeated measures.
shows the relationship between $J_H$ and pH$_i$ in this protocol. $J_H$ values at pH 6.90 and 6.95 were significantly depressed in both lines of AT$_2$ TG mice compared with WT mice ($P < 0.01$, respectively). The calculation of $J_H$, as well as the inference that it predominantly reports activity of the NHE, requires assessment of $i$Na (see MATERIALS AND METHODS). There were no significant differences in $i$Na among the groups.

The protein levels of NHE were similar between WT and AT$_2^{high}$ TG mice (Fig. 7B). These data suggest that the capacity to recruit NHE activation to correct intracellular acidification was impaired in both lines of AT$_2$ TG mice, independent of a change in $i$Na or levels of NHE. Because NO may cause the inhibition of the activity of the NHE in myocytes, we examined the expression level of both eNOS and iNOS. There were no differences in protein levels of eNOS in AT$_2^{low}$, AT$_2^{high}$, and WT mice (118 ± 9% and 106 ± 9% vs. 100 ± 5%, $P = $ not significant; data not shown). iNOS protein was not detected in LV tissues in AT$_2^{high}$, AT$_2^{low}$ TG, or WT mice.

**DISCUSSION**

AT$_2$ is very sparse in normal postnatal ventricle but the proportion of AT$_2$ to AT$_1$ is upregulated in chronic heart failure. This study was designed to directly test the hypothesis that chronic AT$_2$ expression depresses myocardial contractility by using TG mice with overexpression of AT$_2$ specific to ventricular myocytes driven by the MLC2v promoter. In addition, the use of two lines of AT$_2$ TG mice permitted the analysis of effects of differing levels of AT$_2$ expression. Myocytes from AT$_2$ TG mice exhibited 1) impaired capacity to increase contractility at faster depolarization rates, 2) impaired inotropic response to isoproterenol and ANG II, and 3) depressed capacity to correct intracellular acidification. In this regard, a novel and unexpected finding was that ANG II promoted a negative inotropic effect in AT$_2$ TG myocytes. Whereas the response to isoproterenol did not distinguish the two lines of TG mice, the response to ANG II was related to
the level of AT2 overexpression. The negative inotropic response to ANG II was also observed in AT2 TG myocytes at a young age when heart failure was not yet evident. This study demonstrated that chronic overexpression of AT2 in ventricular myocytes has the potential to depress three key components of myocyte contractile physiology that contribute to integrated cardiac reserve during exercise and stress in vivo.

Contractile dysfunction in AT2 TG myocytes. The present study corroborated reports that AT2 expression is negligible in normal adult ventricular myocytes (11, 32, 34). A new observation is that contractile dysfunction is depressed in isolated myocytes from mice with chronic ventricle-specific AT2. Cell size was increased and fractional cell shortening and peak systolic [Ca^{2+}i] were severely depressed in AT2^{high} TG myo-

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**Fig. 5.** Contractile dose response to ANG II (1–100 nmol/l) in LV myocytes from AT2^{high} TG, AT2^{low} TG, and WT mice between 18 and 22 wk of age. A: relationship between ANG II concentration and fractional cell shortening in all groups. B: relationship between ANG II concentration and peak systolic [Ca^{2+}i]. The contractile response to ANG II was blunted in AT2^{high} TG myocytes. The negative inotropic effect was induced in AT2^{low} TG myocytes. C and D: response to ANG II in the presence of losartan (1 μmol/l) or PD-123319 (100 nmol/l) in WT (C) and AT2^{high} TG (D) myocytes. Both positive and negative inotropic effects were abolished with losartan but not PD-123319; n = 13–15 experiments from 6 hearts (WT), 9–11 experiments from 7 hearts (AT2^{low} TG), and 8 or 9 experiments from 6 hearts (AT2^{high} TG). Statistical analysis was performed by ANOVA with repeated measures. *P < 0.05, **P < 0.01, ***P < 0.001 between indicated groups.
Fig. 6. Contractile dose response to ANG II (1–100 nmol/l) in LV myocytes from AT2\textsuperscript{high TG}, AT2\textsuperscript{low TG}, and WT mice between 4 and 5 wk of age. A: relationship between ANG II concentration and fractional cell shortening in all groups. B: relationship between ANG II concentration and peak systolic $[\text{Ca}^{2+}]$. The contractile response to ANG II was blunted in AT2\textsuperscript{low TG} myocytes. The negative inotropic effect of ANG II stimulation was observed in AT2\textsuperscript{high TG} myocytes. C and D: response to ANG II in the presence of losartan (1 μmol/l) or PD-123319 (100 nmol/l) in WT (C) and AT2\textsuperscript{high TG} (D) myocytes. Both positive inotropic effects in WT and negative inotropic effects in AT2 TG myocytes were abolished with losartan but not PD-123319; $n = 11$ or 12 experiments from 9 hearts (WT), 9–11 experiments from 6 hearts (AT2\textsuperscript{low TG}), and 9 or 10 experiments from 8 hearts (AT2\textsuperscript{high TG}). Statistical analysis was performed by ANOVA with repeated measures. *P < 0.05, ***P < 0.001 between indicated groups.
cytes, whereas mild depression without any change in cell size was observed in AT2\textsuperscript{low} TG myocytes. Frequency-dependent contractile reserve, assessed as the capacity to augment both myocyte contraction and peak systolic [Ca\textsuperscript{2+}], at faster pacing rates, was also severely depressed in AT2\textsuperscript{high} TG myocytes. In AT2\textsuperscript{low} TG myocytes, the response to the pacing challenge was intermediate between that of AT2\textsuperscript{high} TG and WT myocytes.

Although multiple factors contribute to contractile reserve, the ratio of SERCA2a to its inhibitory protein, PLB, is a determinant of cardiac contractility and SR function (7). We previously demonstrated (15) that a depressed ratio of SERCA2 to PLB in hypertrophied myocytes is associated with impaired capacity to augment SR Ca\textsuperscript{2+} stores at high work states, emphasizing the importance of the regulation of SR Ca\textsuperscript{2+} load on contractile reserve. In the present study, the expression of PLB was increased with preserved protein levels of SERCA2a. Prior experiments showed that the overexpression of PLB is sufficient to cause depressed contractility with decreased peak systolic Ca\textsuperscript{2+} level and prolonged time for decay in Ca\textsuperscript{2+} signal (19). Together, the slow relaxation and decay in [Ca\textsuperscript{2+}]; and depressed contractile reserve in myocytes from AT2\textsuperscript{high} TG mice are likely to be related to depressed SR Ca\textsuperscript{2+} uptake capacity. In addition, isoproterenol failed to restore the depressed contractile function in both lines of AT2 TG mice. An abnormal contractile response to β-adrenergic agonists is ubiquitous in heart failure, associated with multiple defects in the signaling pathway, and found in multiple human and animal models of heart failure (6, 10). Thus the depressed response to isoproterenol in our model is likely to be a secondary nonspecific adaptation to heart failure. Future studies will be needed to fully examine the regulation of β-adrenergic signaling, and its age dependence, in this transgenic model.

**Negative inotropic response to ANG II.** A second major finding in this study is that ANG II induced a negative inotropic response in AT2 TG myocytes compared with a positive inotropic effect in WT myocytes, which was mediated by an increase in intracellular Ca\textsuperscript{2+}. In contrast with the isoproterenol response, the depressed inotropic response to ANG II was related to the level of AT2 overexpression and distinguished myocytes from the two lines of AT2 TG mice. Unexpectedly, the negative inotropic effects of ANG II in the AT2 TG myocytes were inhibited by the AT1 antagonist losartan but not by the AT2 antagonist PD-123319. In addition, the direct AT2 agonist CGP-42112A did not depress contractility in AT2 TG myocytes. Furthermore, the negative inotropic response to ANG II was also observed in myocytes from young AT2\textsuperscript{high} TG mice, when heart failure was not yet developed. These observations suggest that the abnormal contractile response to ANG II is related to myocyte-specific expression of AT2 rather than a nonspecific change associated with late heart failure.

Together, these observations support the hypothesis that the striking negative inotropic effects of ANG II in AT2 TG myocytes were mediated by an interaction of AT1 and AT2 independent of the acute activation of AT2 in isolation. These findings are consistent with a discrepancy between gene manipulation and pharmacological blockade reported by Adachi et al. (2). In that study, although the knockout of AT2 gene in mice caused early mortality after experimental infarction, pharmacological blockade of AT2 receptor with PD-123319 did not duplicate this finding. Recently, AbdAlla et al. (1) suggested that AT2 is a unique G protein receptor that acts as an AT1-specific antagonist independent of direct ANG II binding.

**Table 3. Basal intracellular pH in myocytes**

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>AT2\textsuperscript{low} TG</th>
<th>AT2\textsuperscript{high} TG</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.25±0.05</td>
<td>7.20±0.03</td>
<td>7.19±0.04</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SE intracellular pH (pH\textsubscript{i}) for n = 10 experiments from 3 hearts (WT), 15 from 3 hearts (AT2\textsuperscript{low} TG), and 12 from 3 hearts (AT2\textsuperscript{high} TG). Measurements were obtained at a pacing frequency of 0.5 Hz.

**Graphs**

Fig. 7. A: relationship between the net influx of H\textsuperscript{+} (J\textsubscript{H}) and the pH at which it occurred. Statistical comparison was performed with J\textsubscript{H} values between pH 6.9 and 7.2. J\textsubscript{H} in the presence of intracellular acidification was depressed in AT2 TG myocytes at pH 6.9 and 6.95; n = 13 experiments from 4 hearts (WT), 12 experiments from 3 hearts (AT2\textsuperscript{low} TG), and 16 experiments from 5 hearts (AT2\textsuperscript{high} TG). **p < 0.01 compared with WT. B: representative data of LV Na\textsuperscript{+}/H\textsuperscript{+} exchanger-1 (NHE-1) protein levels from WT and AT2\textsuperscript{low} TG myocytes. C: protein levels of NHE-1 in WT and AT2\textsuperscript{high} TG myocytes (n = 5). Expression levels of NHE-1 were similar between WT and AT2\textsuperscript{high} TG myocytes.
to AT2 and activation of AT2 downstream signaling. They reported that AT2 formed heterodimers with AT1 in cultured cell lines, as well as in human myometrial cells, and that ANG II in the presence of AT1-AT2 heterodimerization suppressed inositol phosphate generation mediated by AT1 independent of the ANG II activation of AT2. In cardiomyocytes, such heterodimerization and depression of inositol phosphate generation might be expected to depress contractile function and systolic intracellular Ca2+. The hypothesis of AbdAlla et al. (1) is consistent with our findings that the ANG II-induced negative inotropic effect was related to the expression level of AT2 in the two lines of TG mice but was independent of the actions of direct AT2 antagonists and agonists. Whereas our preliminary confocal microscopic analyses are not sufficient to show molecular dimerization, these studies do suggest partial colocalization of AT1 with AT2. Further studies of membrane ultrastructure will be needed to demonstrate colocalization of AT1 and AT2 and elucidate effects on AT1 signaling.

Impaired activation of NHE. Contractile reserve of the normal heart also requires the capacity to rapidly correct intracellular acidification. At higher work states, increased proton generation may occur from high metabolic state or transient ischemia. pH, a major determinant of myofilament Ca2+ sensitivity (8), is strictly regulated by two sarcoplasmic ion transporters: the NHE and the Na+/H+ cotransporter (30). In isolated myocytes, the NHE contributes to ~60% of total proton efflux (22). Basal pH, was unchanged in AT2 TG and WT myocytes. However, proton transport by the NHE in response to abrupt intracellular acidification was impaired in both TG lines. Thus, under conditions of sudden intracellular acidification, the recruitment of NHE activity is impaired in myocytes with AT2 expression, which has the potential to suppress correction of acidification and exacerbate contractile dysfunction. Our observations are consistent with a previous report that AT2 signaling in vascular smooth muscle cells induces intracellular acidification via inhibition of NHE activity (31).

Several mechanisms may be contributory. NHE activation depends in part on phosphorylation by protein kinase C and MAP kinase (18, 27). It has been demonstrated that AT2 activates several phosphatases (13, 25) and inhibits MAP kinase-dependent phosphorylation in rat myocytes in vitro (9). Increased NO production (17) is an additional mechanism that could inhibit NHE activity in AT2 TG myocytes. AT2 receptor activation stimulates eNOS expression in cultured cardiomyocytes (28) and enhances NO production in vascular smooth muscle cells (31). However, in the present study, there was no increase in LV eNOS protein levels in AT2 TG compared with WT myocytes, and iNOS was not detectable in any group.

In conclusion, this study demonstrates that ventricle-specific AT2 expression is associated with depression of three distinct components of myocyte contractile function that contribute to integrated cardiac performance in vivo. These defects include the capacity to 1) augment Ca2+--dependent contractility in response to an increase in heart rate, 2) respond to inotropic stimulation by ANG II, and 3) rapidly correct intracellular acidification. This study is limited by known differences between the regulation of contractility in mouse and human myocytes (5). In addition, future in vitro studies will be required to determine whether the mechanisms include AT2 heterodimerization with AT1 or activation of other signaling pathways. Together, these findings support the hypothesis that chronic overexpression of AT2 in the ventricle may directly promote depressed contractile function and contribute to the progression of heart failure.

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