Gain-of-function mutant of angiotensin II receptor, type 1A, causes hypertension and cardiovascular fibrosis in mice

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The role of the renin-angiotensin system has been investigated by overexpression or inactivation of its different genes in animals. However, there is no data concerning the effect of the constitutive activation of any component of the system. A knockin mouse model has been constructed with a gain-of-function mutant of the Ang II receptor, type 1A (AT1A), associating a constitutively activating mutation (N111S) with a C-terminal deletion, which impairs receptor internalization and desensitization. In vivo consequences of this mutant receptor expression in homozygous mice recapitulate its in vitro characteristics: the pressor response is more sensitive to Ang II and longer lasting. These mice present with a moderate (~20 mmHg) and stable increase in BP. They also develop early and progressive renal fibrosis and cardiac fibrosis and diastolic dysfunction. However, there was no overt cardiac hypertrophy. The hormonal parameters (low-renin and inappropriately normal aldosterone productions) mimic those of low-renin human hypertension. This new model reveals that a constitutive activation of AT1A leads to cardiac and renal fibrosis in spite of a modest effect on BP and will be useful for investigating the role of Ang II in target organs in a model similar to some forms of human hypertension.

Introduction

The Ang II receptor, type 1 (AT1), is a G protein–coupled receptor (GPCR) that transduces the main physiological actions of the renin-angiotensin system (RAS) in target cells. The major signaling events following agonist binding to this receptor are activation of phospholipase C via a Gq protein, mobilization of the calcium intracellular stores, and activation of protein kinase C. MAP kinase and Jak/STAT pathways are also activated and may participate in the hypertrophic actions of Ang II (1). There are 2 AT1 subtypes in rodents, AT1A and AT1B (2, 3); the tissue distribution of the 2 subtypes (4) and knockout experiments in mice (5–7) indicate that cardiovascular, renal, and adrenal actions of Ang II are mainly mediated by AT1A. The unambiguous role of AT1 in controlling BP is evidenced in numerous animal models. For example, the knockout of AT1A in mouse reduces BP (5, 6), and its overexpression by different constitutively active mutations, one of the most active is the N111S. These AT1A leads to cardiac and renal fibrosis in mice despite a modest effect on BP and will be useful for investigating the role of Ang II in target organs in a model similar to some forms of human hypertension (9).

The concept of constitutive activation is an important breakthrough in the understanding of GPCR molecular functions: point mutations stabilize the receptor in an active conformation independent of ligand, resulting in a permanent activation of the signaling pathways (10). Several human genetic diseases appear to be consequences of such GPCR gain-of-function mutant expression (11, 12). Therefore, it was logical to investigate whether cases of human hypertension are associated with such activating mutations of AT1. However, no mutation of AT1 coding sequence had previously been identified in hypertension or, more particularly, in primary hyperaldosteronism (13, 14). This absence of clearly identified gain-of-function mutations of AT1 in humans prompted us to map the mutations of the rat AT1A, that cause constitutive activity by screening a randomly mutated library of the receptor with a functional assay (15). The next step was to develop an animal model expressing a gain-of-function mutant of AT1A in humans.

The choice of the mutation(s) was directed by the initial in vitro characterization of these constitutively activated mutants. All these AT1A mutants are slightly constitutively active when compared with the WT receptor but are far from being fully active. Therefore, they can still be activated by agonists. Among the different constitutively active mutations, one of the most active is the N111S mutation. This N111S mutation, as other constitutively active mutations, induces constitutive internalization, resulting in intracellular sequestration of most receptor molecules (16). For these reasons, we expected a mild phenotype and added to the N111S mutation a C-terminal deletion of the receptor, which impairs phosphorylation and internalization, abolishing desensitization and inducing hyper-reactivity to Ang II (17).

Nonstandard abbreviations used: ANF, atrial natriuretic factor; AT1A, Ang II receptor, type 1A; AT1B, wild-type; AT1A, mutant; AT2, WT, AT1A, wild-type; AUC, area under the curve; BNP, brain natriuretic peptide; GPCR, G protein–coupled receptor; PAI1, plasminogen activator inhibitor type 1; RAS, renin-angiotensin system.

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cellular expression of AT₁A bearing both these gain-of-function mutations revealed additivity of constitutive activity and hyper-reactivity to Ang II in vitro (18).

Here, we describe in vivo expression of this gain-of-function mutant in a knockin mouse model and the main characteristics of the phenotype. The constitutive activation of this receptor, associated with impaired internalization, produces a new model of mouse hypertension characterized by normal aldosterone production in the presence of very low plasma renin and the existence of cardiac, vascular, and renal fibrosis.

**Results**

**Generation of mice expressing the AT₁A N111S/Δ329 mutant**

Homozygous mice expressing the gain-of-function AT₁A mutant were generated as described in Figure 1. Specific expression of the AT₁A N111S/Δ329 mutation (AT₁A_MUT), but not AT₁A_WT, was identified by RT-PCR in the major AT₁A-expressing tissues (liver, heart, kidney, adrenal gland, and aorta) of homozygous mutant mice (data not shown). No difference in expression of AT₁B or AT₂ was observed when RNA from those Ang II target tissues of WT and mutant mice were compared using quantitative RT-PCR (Figure 2). Similar results were obtained by in situ hybridization using specific probes for the 3 Ang II receptors (data not shown).

All the phenotypic comparisons were made between homozygous mutant mice and WT littermates at the same age. The mutant mice had significantly lower body weights (10% to 20% lower), first observed in young animals (1 to 3 months old) and persisting throughout the life of the mouse without any clear sex difference (Table 1). An interesting and unexpected phenotypic trait was the higher concentration of red blood cells (17% higher) and hemoglobin (12% higher) and an increased hematocrit (12% higher), in mutant compared with control mice. These differences seem to correspond to true polycythemia rather than to hemoconcentration because total plasma protein concentration and osmolality were unaffected (Table 2).

**Gain-of-function features of AT₁A_MUT in mice**

*In vitro characterization in tissues and isolated hepatocytes.* The constitutive activity of AT₁A_MUT was first investigated by comparing the phosphorylated state of signaling molecules such as STAT1, STAT3, and ERK1/2 in tissues (liver, heart, and kidney) of AT₁A_WT and AT₁A_MUT expressing mice.
and AT_{1\text{MUT}} mice. Given the modest constitutive activity of the N111S/A329 mutant, it was not surprising to observe only modest changes in basal phosphorylations. In the liver, STAT1 increased 36% (P = NS) and STAT3 increased 63% (P < 0.05), but ERK1/2 phosphorylation did not increase, in AT_{1\text{MUT}} mice (Figure 3A). In heart and kidney, no change was observed in STAT1 or STAT3 (data not shown). In order to gain more insight into the functional properties of AT\textsubscript{1}WT and AT\textsubscript{1\text{MUT}} receptors, their pharmacology and signaling were investigated in freshly isolated hepatocytes (Table 3 and Figure 3). The AT\textsubscript{1\text{MUT}} and AT\textsubscript{1\text{WT}} receptors presented similar expression levels and a similar affinity for Ang II in the liver. The pharmacological profiles of the AT\textsubscript{1\text{MUT}} and AT\textsubscript{1\text{WT}} receptors in the liver were compared: there were no differences in affinity for the peptide analog [Sar1-Ile8]Ang II, but AT\textsubscript{1\text{MUT}} had a lower affinity for the nonpeptide AT\textsubscript{1} antagonist losartan and a higher affinity for CGP42112A (a pseudopeptide agonist for AT\textsubscript{1}) and Ang IV (angiotensins 3–8) (Table 3). Ang IV is a full agonist for these constitutively activated receptors, as indicated by the strong and dose-dependent stimulation of ERK1/2 phosphorylation (Figure 3C). This constitutive activation of AT\textsubscript{1\text{MUT}} in hepatocytes is associated with its hyperreactivity and hypersensitivity to Ang II: compared with the AT\textsubscript{1\text{WT}} receptor, the maximal ERK1/2 phosphorylation in the AT\textsubscript{1\text{MUT}} receptor increased by 2-fold and sensitivity of the response to Ang II was better (50% effective concentration [EC\textsubscript{50}] = 0.39 ± 0.16 nM, AT\textsubscript{1\text{MUT}}; 2.10 ± 0.55 nM, AT\textsubscript{1\text{WT}}; P < 0.05) (Figure 3B). This pharmacological and signaling profile is similar to that reported for the same mutant in transfected cells (18) and reproduces some of the characteristics of constitutively activated AT\textsubscript{1}, which bind inverse agonists with at least 100-fold lower affinity compared with AT\textsubscript{1\text{WT}} receptors (19). Conversely, CGP42112A and Ang IV have a much better affinity and become full agonists for AT\textsubscript{1\text{MUT}} (15).

In vivo vascular responsiveness to Ang II. The BP response to Ang II was analyzed by invasive methods on anesthetized 5-month-old animals (Figure 4). As expected, Ang II increased the BP of AT\textsubscript{1\text{WT}} animals in a dose-dependent manner, with a sharp initial peak after injection followed by a progressive return to basal BP within approximately 3 minutes (Figure 4A). Interestingly, in AT\textsubscript{1\text{MUT}} mice, the initial BP rise was followed by a plateau that lasted for at least 30 minutes, without any return to the basal level (Figure 4A). The cumulative doses of Ang II (0.001–30 μg/kg) raised the systolic BP measured 10 minutes after the last injection by 60 mmHg in AT\textsubscript{1\text{WT}} mice, whereas it increased only 25 mmHg in AT\textsubscript{1\text{WT}} animals. The BP response to Ang II as assessed from the area under the curve in AT\textsubscript{1\text{MUT}} mice (ED\textsubscript{50} = 0.07 ± 0.16 μg/kg) was considerably more sensitive than that in the AT\textsubscript{1\text{WT}} mice (ED\textsubscript{50} = 0.63 ± 0.23 mg/kg; P < 0.05) (Figure 4B). These data suggest strongly that the transduction of the BP response to Ang II via the AT\textsubscript{1\text{MUT}} receptor is more sensitive compared with AT\textsubscript{1\text{WT}} and that this response is not desensitized. This nondesensitized BP response is independent of kidney AT\textsubscript{1}, at least in acute conditions, since binephrectomy does not modify the sustained BP response to Ang II (data not shown). In addition, the specificity of the sustained BP response via AT\textsubscript{1\text{MUT}} is assessed by the clear agonist effect of CGP42112A, which is ineffective in AT\textsubscript{1\text{WT}} mice (Figure 4C) and by a similar BP response to epinephrine in the 2 groups of mice (data not shown).

**Table 1**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Age (mo)</th>
<th>Sex</th>
<th>AT\textsubscript{1\text{WT}}</th>
<th>n</th>
<th>AT\textsubscript{1\text{MUT}}</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW (g)</td>
<td>1–3</td>
<td>M</td>
<td>26.8 ± 1.1</td>
<td>6</td>
<td>22.0 ± 1.4</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>5–7</td>
<td>M</td>
<td>33.3 ± 1.2</td>
<td>3</td>
<td>28.7 ± 2.3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>5–7</td>
<td>F</td>
<td>26.2 ± 0.9</td>
<td>5</td>
<td>20.9 ± 0.4</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>7–11</td>
<td>M</td>
<td>34.3 ± 0.7</td>
<td>10</td>
<td>30.7 ± 0.8</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>7–11</td>
<td>F</td>
<td>29.2 ± 0.4</td>
<td>9</td>
<td>26.5 ± 0.4</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>12–15</td>
<td>M</td>
<td>38.2 ± 2.2</td>
<td>3</td>
<td>33.5 ± 0.8</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>12–15</td>
<td>F</td>
<td>27.8 ± 1.0</td>
<td>6</td>
<td>24.6 ± 0.7</td>
<td>6</td>
</tr>
<tr>
<td>Food intake (g)</td>
<td>5–7</td>
<td>M+F</td>
<td>3.71 ± 0.27</td>
<td>8</td>
<td>3.62 ± 0.32</td>
<td>8</td>
</tr>
<tr>
<td>Water intake (ml)</td>
<td>5–7</td>
<td>M+F</td>
<td>3.43 ± 0.22</td>
<td>8</td>
<td>3.17 ± 0.44</td>
<td>8</td>
</tr>
<tr>
<td>Diuresis (ml)</td>
<td>5–7</td>
<td>M+F</td>
<td>1.50 ± 0.11</td>
<td>8</td>
<td>1.09 ± 0.13</td>
<td>8</td>
</tr>
</tbody>
</table>

\( ^{a}P < 0.05, ^{b}P < 0.01 \) compared with AT\textsubscript{1\text{WT}}. BW, body weight.
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The consequences for the RAS of the expression of this AT1A MUT on kidney structure and functions: urinary samples were collected from 6- to 7-month-old animals in metabolic cages, plasma was sampled, and renal morphology was analyzed.

The 24-hour diuresis of AT1A MUT animals was lower than AT1A WT (Table 1). This was associated with a higher urinary osmolality but a normal plasma osmolality (Table 2).

After correction for body weight and 24-hour diuresis, creatinine excretion in mutant mice was significantly lower than in AT1A WT mice (Figure 7A), whereas plasma creatinine concentration was normal and plasma urea was slightly but significantly higher (Table 2). There were no obvious tubular pathology or major plasma or urinary electrolyte abnormalities (Figure 7B and Table 2).

These moderate functional changes were associated with periglomerular and perivascular fibrosis without major changes in glomerular or tubular morphology (Figure 7C). Confirmation of this fibrosis was investigated by measuring expression of 2 molecular markers, collagen type 1 and plasminogen activator inhibitor type 1

Table 2

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Age (mo)</th>
<th>Sex</th>
<th>AT1A WT</th>
<th>n</th>
<th>AT1A MUT</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood Fasting glycemia (mg/dl)</td>
<td>7–11</td>
<td>M+F</td>
<td>84 ± 1.1</td>
<td>19</td>
<td>79.7 ± 1.5</td>
<td>23</td>
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<tr>
<td>Ht (%)</td>
<td>6.5–9</td>
<td>M+F</td>
<td>36.6 ± 1.4</td>
<td>10</td>
<td>41.1 ± 1.2</td>
<td>10</td>
</tr>
<tr>
<td>rbc (10^6/ul)</td>
<td>6.5–9</td>
<td>M+F</td>
<td>7.6 ± 0.2</td>
<td>10</td>
<td>8.9 ± 0.3</td>
<td>10</td>
</tr>
<tr>
<td>Hb (g/dl)</td>
<td>6.5–9</td>
<td>M+F</td>
<td>12.5 ± 0.4</td>
<td>10</td>
<td>14.1 ± 0.4</td>
<td>10</td>
</tr>
<tr>
<td>Total plasma proteins (g/l)</td>
<td>6.5–9</td>
<td>M+F</td>
<td>37.9 ± 1.7</td>
<td>8</td>
<td>38.7 ± 2.4</td>
<td>7</td>
</tr>
<tr>
<td>Osmolality (mOsm/kg H2O)</td>
<td>6.5–9</td>
<td>M+F</td>
<td>254 ± 10</td>
<td>9</td>
<td>252 ± 12</td>
<td>9</td>
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<tr>
<td>Na+ (mmol/l)</td>
<td>6.5–8</td>
<td>M+F</td>
<td>147 ± 2</td>
<td>5</td>
<td>145 ± 2</td>
<td>6</td>
</tr>
<tr>
<td>K+ (mmol/l)</td>
<td>6.5–8</td>
<td>M+F</td>
<td>5.0 ± 0.2</td>
<td>5</td>
<td>5.8 ± 0.8</td>
<td>6</td>
</tr>
<tr>
<td>Creatinine (μmol/l)</td>
<td>6.5–9</td>
<td>M+F</td>
<td>23.5 ± 1.7</td>
<td>8</td>
<td>22.7 ± 0.8</td>
<td>7</td>
</tr>
<tr>
<td>Urea (mmol/l)</td>
<td>6.5–9</td>
<td>M+F</td>
<td>7.1 ± 0.5</td>
<td>8</td>
<td>8.7 ± 0.4</td>
<td>7</td>
</tr>
<tr>
<td>Urine Osmolality (mOsm/kg H2O)</td>
<td>5–7</td>
<td>M+F</td>
<td>2044 ± 101</td>
<td>7</td>
<td>2504 ± 166</td>
<td>8</td>
</tr>
<tr>
<td>Na+ (μmol/d)</td>
<td>5–7</td>
<td>M+F</td>
<td>324 ± 26</td>
<td>8</td>
<td>262 ± 29</td>
<td>8</td>
</tr>
<tr>
<td>K+ (μmol/d)</td>
<td>5–7</td>
<td>M+F</td>
<td>433 ± 33</td>
<td>8</td>
<td>333 ± 31</td>
<td>8</td>
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<tr>
<td>Creatinine (nmol/d)</td>
<td>5–7</td>
<td>M+F</td>
<td>8243 ± 506</td>
<td>8</td>
<td>5651 ± 480</td>
<td>8</td>
</tr>
<tr>
<td>Urea (mmol/d)</td>
<td>5–7</td>
<td>M+F</td>
<td>1946 ± 156</td>
<td>6</td>
<td>1386 ± 190</td>
<td>7</td>
</tr>
<tr>
<td>Microalbumin (μg/d)</td>
<td>5–7</td>
<td>M+F</td>
<td>11.1 ± 1.3</td>
<td>8</td>
<td>12.3 ± 1.8</td>
<td>8</td>
</tr>
</tbody>
</table>

αP < 0.05, βP < 0.01, γP < 0.001 compared with AT1A WT. Ht, hematocrit; Hb, hemoglobin.

elevation was moderate (~20 mmHg) and persisted with similar severity throughout life without progressive aggravation. It was more severe in male mice (23–35 mmHg) than in female mice (11–17 mmHg) mice. Malignant hypertension was not detected at any time, despite careful testing for symptoms. This permanent hypertension was confirmed by invasive measurements of systolic (122 ± 4 mmHg in AT1A MUT mice compared with 95 ± 6 mmHg in AT1A WT mice; P < 0.01) and diastolic BP values (92 ± 6 mmHg in AT1A MUT mice compared with 69 ± 5 mmHg in AT1A WT mice; P < 0.01) in anesthetized animals.

Pharmacological reduction of BP by AT1 blockers (ARBs) was investigated, despite the fact that ARBs (inverse agonists) present altered affinities for AT1A constitutively activated mutants (19). According to Le et al. (19), candesartan has the highest ARB affinity for the N111S mutant (Kd = 2.49 ± 0.29 nM compared with 0.27 ± 0.07 for the AT1A WT receptor) and losartan has the lowest affinity for the mutant receptor (Kd = 548 ± 140 nM). Candesartan treatment (10 mg/kg/d) during 25 days reduced (by approximately 30 mmHg) BP of AT1A MUT animals to normal levels (Figure 5B).

However, losartan treatment (30 mg/kg/d or 150 mg/kg/d) for 2 weeks significantly reduced the BP of AT1A WT animals (by 11 mmHg and 20 mmHg for the respective doses) but had no effect on AT1A MUT animals (data not shown).

Renal consequences of hypertension and RAS alterations

We investigated the pathophysiological consequences of the expression of AT1A MUT on kidney structure and functions: urinary samples were collected from 6- to 7-month-old animals in metabolic cages, plasma was sampled, and renal morphology was analyzed.

The 24-hour diuresis of AT1A MUT animals was lower than AT1A WT (Table 1). This was associated with a higher urinary osmolality but a normal plasma osmolality (Table 2).

After correction for body weight and 24-hour diuresis, creatinine excretion in mutant mice was significantly lower than in AT1A WT mice (Figure 7A), whereas plasma creatinine concentration was normal and plasma urea was slightly but significantly higher (Table 2). There were no obvious tubular pathology or major plasma or urinary electrolyte abnormalities (Figure 7B and Table 2).

These moderate functional changes were associated with periglomerular and perivascular fibrosis without major changes in glomerular or tubular morphology (Figure 7C). Confirmation of this fibrosis was investigated by measuring expression of 2 molecular markers, collagen type I and plasminogen activator inhibitor type 1.

Table 3

<table>
<thead>
<tr>
<th>Experiment type</th>
<th>Parameter</th>
<th>AT1A WT</th>
<th>AT1A MUT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saturationα</td>
<td>Kd (nM)</td>
<td>0.54 ± 0.03</td>
<td>0.58 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>Bmax (fmol/mg)</td>
<td>12.6 ± 2.4</td>
<td>11.0 ± 2.3</td>
</tr>
<tr>
<td>Competitionβ</td>
<td>Kd (nM)</td>
<td>0.24 ± 0.21</td>
<td>0.41 ± 0.19</td>
</tr>
<tr>
<td></td>
<td>[Sar1-Ileu8] Ang II</td>
<td>14.5 ± 3.9</td>
<td>1857.5 ± 728.5</td>
</tr>
<tr>
<td></td>
<td>Losartan</td>
<td>1375.7 ± 383.5</td>
<td>74.8 ± 20.9</td>
</tr>
<tr>
<td></td>
<td>CGP42112A</td>
<td>207.8 ± 22.5</td>
<td>11.2 ± 3.5</td>
</tr>
<tr>
<td></td>
<td>Ang IV</td>
<td>190 ± 7.8</td>
<td>38.7 ± 3.8</td>
</tr>
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</table>

αParameters of [125I]Ang II binding calculated from saturation experiments.

βKd calculated from competition experiments of [125I]Ang II with the indicated compounds. Results are expressed as mean ± SEM of 3 independent experiments, in which each point was performed in triplicate. γP < 0.05 compared with AT1A WT.
(PAI1), using quantitative RT-PCR (Figure 7D). Despite a 3-fold increase in these markers in AT1A MUT mice, this difference was not significant due to an important variability of the markers in AT1A MUT mice. The origin of this variability is not linked to age but may be linked to gender, and it should be further investigated. A moderate but significant hypertrophy of the small renal arteries was also observed (Figure 8A).

**AT1A MUT mice present with progressive cardiac fibrosis**

Morphological and echocardiographic investigations of the cardiac structure and function in 5- and 12-month-old AT1A MUT and AT1A WT mice were performed. The AT1A MUT animals did not present any major cardiac hypertrophy. The heart weight/body weight ratio was unchanged in 5-month-old animals and in 12-month-old females (Figure 8B). However, the ratio was slightly but significantly higher in 12-month-old AT1A MUT males compared with AT1A WT males (data not shown). These changes in heart weight were not associated with any increase in atrial natriuretic factor (ANF) expression, but a clear (>3-fold) and significant increase of brain natriuretic peptide (BNP) expression was observed in the heart of AT1A MUT mice (Figure 8C).

The absence of obvious cardiac hypertrophy in mutant mice was confirmed by echocardiography. Left ventricle mass was the same in mutant and AT1A WT mice at both 5 and 12 months of age (Table 4). Echocardiography detected a mild, late decrease in contractility in AT1A MUT mice as assessed by tissue Doppler imaging (Table 4); the ejection fraction was not significantly lower, but there was mild left ventricle remodeling, as shown by the higher left ventricle end-diastolic diameter relative to body weight in AT1A MUT mice. However, a clear, early alteration in diastolic left ventricle function was detected (Table 4) with variables associated with myocardial relaxation being abnormal. In 12-month-old mice the abnormal diastolic function was accompanied by high left ventricle filling pressure, as depicted by the ratio of the maximal blood velocity of early left ventricle inflow to the maximal velocity of early diastolic motion of the mitral annulus, with relatively conserved systolic function, the hallmark of diastolic heart failure.

Morphological analysis of serial heart slices unambiguously showed the presence of a cardiac fibrosis in AT1A MUT mice (Figure 8D), without cardiomyocyte hypertrophy or inflammation. This fibrosis predominated in pericoronary area but was also observed in interstitial spaces. It was not detected until 3 months of age, but it
became apparent in 4-month-old mutant mice and progressed with time to become evident at 1 year without any sex preference. This cardiac fibrosis was confirmed at the molecular level, with an important (>3-fold) and significant increase of collagen α1 expression, whereas only a limited and nonsignificant increase of PAI1 expression was observed in 5-month-old AT1A MUT mice (Figure 8E).

Discussion
Expression of a gain-of-function AT1A MUT at its physiological sites in the mouse results in a permanent, nonmalignant form of hypertension characterized by a normal aldosterone level but low-renin secretion. This permanent hypertension is unusual because of its early development, the absence of aggravation during mouse life, its moderate severity, and its predominance in males. The roles of various genes of the RAS that are potentially involved in the control of BP (renin, angiotensinogen, angiotensin-converting enzyme, and AT1 genes) has been extensively investigated both by overexpression and by inactivation in mice and rats (20–26). Inactivation of the Agrp1a gene (27) but not the Agrp1b (7) or the Agrp2 (28, 29) genes, causes a BP decrease, and the duplication of Agrp1a gene results in a moderate increase of BP in female mice (8). Sex differences in BP changes were also observed in Ren1d KO (21) and Ace KO (20) female mice, which had decreased BP, but these apparently discordant differences were never explored further. These data confirm that quantitative alterations of RAS gene expression modulate BP. However, there are no data about the effect of constitutive activation of any of the RAS components on BP or cardiovascular or renal functions.

In the present study, the observed phenotype is a result of qualitative, not quantitative, functional alterations of AT1A. Indeed,
Agtr1a, Agtr1b, and Agtr2 levels of expression are unchanged in mutant mouse tissues as assessed by quantitative RT-PCR in several target organs of Ang II and Ang II binding and in situ hybridization. These qualitative alterations of AT₁A associate 2 gain-of-function mutations.

The first mutation is an N111S point mutation that induces partial constitutive activation of AT₁A. Constitutive activation of AT₁A is confirmed in vivo in AT₁A MUT mice by several experimental data: (a) pharmacological and signaling profiles of AT₁A that are similar in hepatocytes from AT₁A MUT mice and cells transfected with different constitutively active AT₁A mutants (15, 18); (b) increase of STAT1, STAT3, and ERK1/2 basal phosphorylation in some Ang II target tissues; (c) correction of hypertension with the inverse agonist candesartan, which has high affinity for AT₁A MUT (19); (d) and full agonist effect of CGP42112A on AT₁A MUT mouse BP, as previously shown in vitro on constitutively active mutants (15). Altogether, these results sustain the hypothesis that, at least in part, the mouse phenotype is a result of constitutive activation of the receptor. This animal model, in addition to cellular models expressing the AT₁A MUT receptor, could thus help in the evaluation of other new and more potent agonists or antagonists. The AT₁A MUT mouse is the first animal model expressing a constitutively active GPCR in a physiological context. Previous reports have described overexpression of constitutively active mutants of the α1B and β2 adrenergic receptors in the heart (30, 31) and the parathyroid hormone receptor in the growth plate (11). Surprisingly the gain-of-function phenotype was often replaced by a loss-of-function phenotype as a result of cellular sequestration or toxicity of the GPCR mutant. To avoid constitutive internalization and desensitization of the constitutively active AT₁A previously described in cellular models (16), we added a second mutation, i.e., a C-terminal deletion (Δ329). In absence of the C-terminal tail, the receptor cannot be phosphorylated, does not interact with β-arrestins, is not internalized, and is not desensitized (17). The in vivo consequences of this deletion confirmed the observations in cellular systems. The hypertensive response to Ang II was amplified and long lasting in AT₁A MUT animals. This sustained response to Ang II strongly suggests that the mutant receptor is not turned off after Ang II activation. Other reports indicated the possibility of desensitization alterations of GPCRs such as β-adrenergic receptors in heart failure (32), but to our knowledge the present knockin (AT₁A MUT) is the first animal model in which the physiological consequences of the expression of a non-desensitized GPCR could be investigated.

Hypertension of AT₁A MUT mice is associated with low plasma renin levels, a consequence of a decreased renal renin synthesis, as shown by in situ hybridization and low plasma angiotensin levels. In contrast, there is no alteration in aldosterone synthesis, plasma and urinary aldosterone are normal, there are no major changes in electrolyte balance, and notably, there is normal kalemia. Such a discrepancy between renin production and aldosterone production has already been observed in Agtr1a KO mice (high renin and normal aldosterone levels) (33). A possible explanation for the lack of aldosterone overproduction is the particular status of the zona glomerulosa and the zona fasciculata.
The clear dipsogenic effect of the mutant receptor. This suggests that the presence of an inappropriate vasopressin secretion, and there is no evidence of an inappropriate vasopressin secretion, and there is no clear dipsogenic effect of the mutant receptor. This suggests that the decrease in circulating Ang II. The result may be a normal aldosterone production, which is nevertheless inappropriate in view of the low-renin values. Alternatively the normal levels of aldosterone could be the result of other RAS-independent factors regulating aldosterone production. This phenotype, together with the hyper-sensitivity to Ang II infusion, resembles that classically described in primary hyperaldosteronism, in which there is an abnormal renin/aldosterone ratio (34). We and others have tried unsuccessfully to identify mutations of the Agtr1 gene in aldosterone-producing adenomas (13, 14), but other forms of low-renin hypertension with or without hyperaldosteronism should be screened.

This ATaMUT mouse is an interesting model in which to investigate the renal modifications that follow receptor activation. Such a model may help to define the roles of renal and extrarenal tissues in the development of hypertension. A recent, elegant study by Crowley et al. demonstrated the additive roles of kidney and systemic tissues in BP regulation using a renal cross-transplantation strategy (35). Similar experiments in ATaMUT mice would further define these distinct roles in the regulation of BP via the RAS.

Ang II regulates water balance by both central (dipsogenic effects) and renal (vasopressin secretion, urine concentration) actions. The ATaMUT mice had lower diuresis and higher urinary osmolality than controls. The opposite (higher diuresis and lower urinary osmolality) has been observed in ATaKO mice (36). Normal plasma natremia in ATaMUT mice excludes the presence of an inappropriate vasopressin secretion, and there is no clear dipsogenic effect of the mutant receptor. This suggests that ATaMUT acts on water homeostasis mostly by urine concentration in the kidney (33).

Ang II also controls the sodium balance by regulating renal hemodynamics and stimulating sodium reabsorption in the renal tubules directly or indirectly via aldosterone secretion. No major modification of the sodium balance was detected in ATaMUT mice on a normal sodium diet, but further studies involving various sodium diets and water intakes will be necessary to precise the link between sodium balance and hypertension in these animals.

The consequences of ATa activation on end organ damage were investigated in ATaMUT mice. Cardiovascular hypertrophy, as assessed by morphological and echographical analyses, is moderate and late in mutant mice. It is observed especially in old male mice, suggesting a predominant role of hypertension in its development. Therefore, ATa activation in the heart seems to have little physiological involvement in cardiomyocyte hypertrophy. This result strongly contrasts with other experimental data, showing that ATa-specific overexpression in the heart of transgenic mice (37–39) induces massive cardiac hypertrophy with conduction defects but without hypertension. However, part of this phenotype may be the result of a nonspecific effect of ATa overexpression in cardiomyocytes.

The major cardiovascular modification in ATaMUT mice is fibrosis. This fibrosis appears in early adulthood (4 months), progresses throughout the life of the mouse, is predominantly peri-vascular but is also interstitial, and is associated with an increased expression of collagen α1. Cardiac fibrosis parallels the functional cardiac alterations that occur early in ATaMUT mice (5 months of age), and this phenotype is similar to that observed in human cardiac fibrotic diseases, i.e., alteration of diastolic function, with subsequent evolution toward diastolic heart failure. The results in ATaMUT mice point to a direct action of Ang II via ATa on extracellular matrix regulation in cardiovascular fibroblasts, as suggested by others (40). Further investigation of the ATaMUT model is needed to delineate the direct physiological effects of ATa activation in cardiovascular hypertrophy, fibrosis, and remodeling, independent of hypertension and other factors such as aldosterone.

In conclusion, this description of what we believe to be the first animal model expressing a gain-of-function mutation of ATa in a physiological context may provide new insight into the pathophysiology and therapy of human hypertension. Our findings suggest that such genetic defects in humans, if they exist, contribute to high aldosterone-low-renin forms of hypertension. The prevalence of these forms of hypertension, as assessed by aldosterone/renin ratio measurements, is high (34). This model also reveals the importance of ATa activation in cardiovascular remodeling and therefore sustains the usefulness of RAS inhibition in these disorders.
Methods

Generation of $\text{AT}_{1A}\text{MUT}$ mice

The targeting $\text{AT}_{1A}$-N111S/Δ329 construct was derived from a 7-kb SpeI(S1)-SacI(A2) genomic fragment contained in a commercial BAC clone (catalog no. FBAC4432; Incyte Genomics Inc.). This 7-kb fragment contains the entire exon 3 of the mouse $\text{Agtr1a}$ gene flanked with 3 kb of intron 2 at its 5′ end and 2 kb of noncoding sequence at its 3′ end (Figure 1). Exon 3 is composed of 5′ untranslated sequence (60 bp), the entire coding sequence, and the 3′ untranslated sequence (830 bp). This 7-kb fragment was digested with SacI, and 4-kb SpeI(S1)-SacI(A1) (fragment 1) and 3-kb SacI(A1)-SacI(A2) (fragment 2) were inserted into a pBlueScript vector (Figure 1A). The N111S mutation and C-terminal deletion of the $\text{AT}_{1A}$ coding sequence were introduced into a 1.5-kb XbaI(X)-SacI(A1) fragment derived from fragment 1 by site-directed mutagenesis using sequential PCR, as previously described (18). The mutated 1.5-kb XbaI/SacI fragment was reintroduced into fragment 1 in place of the corresponding WT fragment, and fragment 2 was reintroduced into this construction at the SacI site.

The hygromycin resistance gene flanked by loxP sites was inserted into the correct orientation into the BgIII site upstream from the hygromycin cassette ligated between the Nrul and Notl sites. This construct was verified by DNA sequencing and restriction mapping.

The final construct was digested with BamHI and KpnI to eliminate plasmid DNA sequences and was electroporated into mouse SV129 ES cells (clone CK35; a gift from C. Kress, Institut Pasteur, Paris, France). Transfected cells were selected with hygromycin 24 hours after electroporation. The DNA of 400 resistant ES cell clones was analyzed by Southern blotting using 2 probes, an internal $\text{AT}_{1A}$ sequence probe (probe 1) and an external 3′ fragment probe (probe 2). Digestion of the genomic DNA with SpeI resulted in a 4.8-kb band for the recombinant gene and a 9-kb band for the endogenous WT gene with probe 1. SacI digestion resulted in a 5-kb band for the recombinant gene and a 3-kb band for the endogenous gene recognized by probe 2 (Figure 1, A and B).

Two appropriate clones were identified by Southern blot (1 positive ES cell clone is shown in Figure 1B) and PCR (data not shown) and presented a normal karyotype. They were microinjected into C57BL/6 blastocysts, which were then implanted into pseudopregnant mice. Several chimeric animals from 2 different lines were able to transmit the transgene to their progeny. Heterozygous progeny was obtained by successive backcrosses to C57BL/6 females. Homozygous and heterozygous animals were obtained into the BgIII site. The BgIII-BgIII fragment was then reintroduced in the correct orientation into the BgIII site upstream from the hygromycin cassette ligated between the Nrul and Notl sites. This construct was verified by DNA sequencing and restriction mapping.

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Figure 8

Cardiovascular characterization of $\text{AT}_{1A}\text{MUT}$ and $\text{AT}_{1A}\text{WT}$ mice. (A) Vascular morphology was assessed by morphometric analysis of the wall thickening ratio (S/L) in renal arteries in 5-month-old $\text{AT}_{1A}\text{WT}$ (white bar) and $\text{AT}_{1A}\text{MUT}$ (black bar) littermates. Three mice were studied in each group, and a mean of 20 arteries were measured in each mouse. *$P < 0.05$ compared with $\text{AT}_{1A}\text{WT}$. (B) Cardiac hypertrophy was assessed by the ratio of heart weight to body weight (HW/BW) in 6- to 10-month-old $\text{AT}_{1A}\text{WT}$ (white triangles) and $\text{AT}_{1A}\text{MUT}$ (black circles) littermates. (C) Quantitative RT-PCR of ANF and BNP mRNA in $\text{AT}_{1A}\text{WT}$ (white bars) and $\text{AT}_{1A}\text{MUT}$ (black bars) hearts. (D) Myocardial sections from 5-month-old $\text{AT}_{1A}\text{WT}$ and $\text{AT}_{1A}\text{MUT}$ mice were stained with Sirius red. Scale bar: 10 μm. (E) Quantitative RT-PCR of collagen αI and PAI1 mRNA in $\text{AT}_{1A}\text{WT}$ (white bars) and $\text{AT}_{1A}\text{MUT}$ (black bars) hearts. **$P < 0.01$ compared with $\text{AT}_{1A}\text{WT}$. 


Freshly isolated hepatocytes were seeded –11

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GeneSnap (version 6.00), and GeneTools software (version 3.07.03) (Syn

cence kit (SuperSignal West Pico; Pierce Biotechnology). ChemiGenius 2,

anti–ERK1/2 antibody (1/1,000; Millipore) and an enhanced chemilumines

tination was analyzed by western blot as previously described (18) using either

37°C with fresh serum-free M199 medium for depletion. Then cells were

cells were washed once with M199 medium and incubated for 3 hours at

on collagen-coated plates at a density of 5

was genotyped using Southern blot analysis of tail biopsies with

in a temperature-controlled environment and had free access to water and

mice were housed in colony cages with a 12-hour light/12-hour dark cycle

Arterial systolic BP and heart rate were measured by tail-cuff plethysmography metho-
d trained conscious mice that had been placed in a warming restrainer (Phymep) as previously described (43). Measurements

were repeated for 4 days after training, and approximately 10 consecutive inflation cycles were performed per day. Successful measure-

ments were averaged to determine the per-

day BP for each mouse. For AT1 blockade assay, candesartan (AstraZeneca) was added to drinking water at 10 mg/kg/d for 25 days. In another set of

experiments, losartan (Teva Pharmaceutical Industries Ltd.) was added to drinking water at 30 mg/kg/d for 15 days and then at 150 mg/kg/d for 15

additional days. BP was measured as described above for the last 3 days of
each treatment period.

In vivo Ang II reactivity

Acute pressor response to Ang II was measured in anesthetized mice. Flex-
ible plastic catheters were introduced into the femoral artery and in the
jugular vein. The arterial catheter was connected to a pressure transducer and a Gould RS 3400 polygraph, and the venous catheter was connected to a syringe for drug injection. The signal was amplified and recorded by a Biopac data acquisition system (MP100; Harvard Apparatus). After at least 30 minutes — necessary to stabilize BP and heart rate — bolus injections of

were repeated for 4 days after training, and

were established for the peak of the BP responses, the area under the curve

from the time of injection to 8 minutes after injection, and the BP difference

tween 2 consecutive Ang II injections. Identical experiments were performed in mice after binephrectomy.

Biochemical measurements

Blood was collected by aorta puncture from anesthetized animals. Hepa-
rinized blood samples were centrifuged at 3,000 g for 5 minutes, and plasma

was collected and frozen.

For urinary determinations, mice were placed individually in metabolic cages for 3 days. Food and water intake and diuresis were measured every day.

Creatinine, urea, protein, Na+, and K+ were determined in plasma and

urine using an Olympus multiparametric autoanalyzer. Hemoglobin, red

blood cell concentration, and hematocrit were determined using a Beck-
man Coulter MAXX automatic analyzer. Plasma and urine osmolalities were measured by Roehling automatic microosmometer.

Plasma renin concentrations were measured by an indirect enzymatic

assay, which determined by RIA the Ang I production of the plasma in

the presence of an excess of the substrate angiotensinogen, as previously described (44). Plasma and urine aldosterone concentrations were deter-
mined by RIA assay as previously described (45). Immunoassay angio-

gene) were used for acquisition and quantifi-
cation of protein phosphorylation.

For basal STAT1, STAT3, and ERK1/2

phosphorylation in tissues, identical amounts of liver, kidney, and heart protein extracts of

AT1WT and AT1MUT mice were analyzed using western blot. Anti-phospho and total

protein antibodies were from Santa Cruz Biotechnology (p-ERK1/2, and STAT3), Millipore (STAT1 and p-STAT3), Cell Signaling Tech-

ology (p-STAT1), and Promega (ERK1/2).

Table 4

Echocardiographic assessment of cardiac phenotypes of AT1WT and AT1MUT mice

<table>
<thead>
<tr>
<th>Age</th>
<th>5 mo</th>
<th>12 mo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AT1WT (n = 8)</td>
<td>AT1MUT (n = 8)</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>550 ± 11</td>
<td>506 ± 13</td>
</tr>
<tr>
<td>LVEDD/BW</td>
<td>0.15 ± 0.01</td>
<td>0.17 ± 0.01</td>
</tr>
<tr>
<td>LV mass/BW (mg/g)</td>
<td>4.17 ± 0.29</td>
<td>4.97 ± 0.32</td>
</tr>
<tr>
<td>EF (%)</td>
<td>80.0 ± 1.8</td>
<td>80.0 ± 0.5</td>
</tr>
<tr>
<td>Sa (cm/s)</td>
<td>3.08 ± 0.14</td>
<td>2.49 ± 0.12</td>
</tr>
<tr>
<td>Spw (cm/s)</td>
<td>3.44 ± 0.27</td>
<td>2.38 ± 0.16</td>
</tr>
<tr>
<td>IVRT (ms)</td>
<td>14.89 ± 0.35</td>
<td>17.2 ± 1.1</td>
</tr>
<tr>
<td>Ea (cm/s)</td>
<td>5.61 ± 0.23</td>
<td>4.99 ± 0.26</td>
</tr>
<tr>
<td>Epw (cm/s)</td>
<td>4.77 ± 0.38</td>
<td>3.87 ± 0.26</td>
</tr>
<tr>
<td>E/Ea</td>
<td>16.7 ± 0.8</td>
<td>17.9 ± 0.5</td>
</tr>
</tbody>
</table>

HR, heart rate; LVEDD/BW, LV end-diastolic diameter normalized to body weight; EF, ejection fraction; Sa, maximal systolic velocity of the mitral annulus; Spw, maximal systolic velocity of the posterior wall; IVRT, isovolumic relaxation time; Ea, early diastolic mitral annular velocity; Epw, early diastolic motion velocity of the posterior wall; E/Ea, ratio of the maximal blood velocity of early LV inflow to the maximal velocity of early diastolic motion of the mitral annulus. *P < 0.05 compared with AT1WT.
tensin was measured in plasma by RIA as previously described (46). This assay measures Ang II, Ang III, and Ang IV together.

**Echocardiographic analysis**

Echocardiography was performed on lightly anesthetized (1% isoflurane in oxygen; Abbot) 5- and 12-month-old mice, as previously described (47). Briefly, left ventricle dimensions and pulse-wave Doppler velocity measurements of the posterior wall (Spw) were obtained from the para-sternal long-axis view. Left ventricle inflow and outflow pulse-wave Doppler velocities, as well as pulse-wave tissue Doppler of the mitral annulus (Ea and Sa) were obtained from apical 5-chamber view.

**Molecular and cellular analyses in Ang II target tissues**

Mice were killed and organs (including heart, aorta, kidney, adrenal gland, and liver) were collected. The organs were briefly rinsed with 0.9% saline to remove blood, blotted dry, and weighed.

For immunoperoxidase staining and in situ hybridization, organs were fixed in 4% buffered paraformaldehyde and embedded in paraffin. Sections mounted for histology were hybridized with cRNA 35S-labeled probes as previously reported (48). Extracellular matrix was stained using Sirius red as described in ref. 49. Samples were examined by optical microscopy.

**Morphometric analysis of arteries.** Multiple nonadjacently sectioned sections from AT1a/WT and AT1a/MUT mice were immunohistochemically labeled with an anti-smooth muscle α-actin antibody. For each α-actin–positive artery, the surface of the media (S) and the longest external diameter (L) were measured as previously described (50). Small arteries with L values between 10 μm and 150 μm were selected. The S:L ratio was used as an index of arterial wall thickness. This ratio was measured for at least 20 different renal arteries for each mouse and was compared between AT1a/WT and AT1a/MUT mice.

**In situ hybridization.** Riboprobe were obtained by in vitro transcription of cloned cDNA for human renin, rat angiotensin-converting enzyme, rat AT1, and AT2, and a specific probe of the mouse CYP11B2 (nucleotides 825 to 901 of NM0009991). After rehydration, microwave heating, and proteinase K digestion, the paraffin-embedded sections were hybridized with 104 cpm/μl of 35S-labeled antisense or sense riboprobe in 30 to 50 μl of hybridization mixture at 50 °C for 16 hours. Samples were washed and then digested with RNase A (20 μg/ml) as previously described (48). The slides were dipped into NTB2 liquid emulsion (Kodak) and stored for 1 to 4 weeks at 4°C in the dark until photographic processing. At the end of the procedure, the slides were examined under a microscope, photographed, and stained with toluidine blue.

**Quantitative RT-PCR.** Quantitative RT-PCR was used to compare the expression of different genes (Agr1a, Agr1b, Agr2, Nppa, Nppb, Col1a1, and serpinel1) in the AT1a/WT and AT1a/MUT mice using the LightCycler technology (Roche Applied Science). The common reference was the cyclophilin gene. Several tissues were analyzed, including liver, heart, kidney, adrenal, and aorta. Total RNA was extracted from these tissues using TRIzol reagents according to the manufacturer’s instructions (Invitrogen), and reverse transcription was performed with the SuperScript II Reverse Transcriptase kit (Invitrogen). All other procedures involving the LightCycler were performed according to manufacturer’s instructions (Roche Applied Science).

**Statistics**

Values are expressed as mean ± SEM. Groups were compared by ANOVA, 2-tailed Student’s t test, or a nonparametric test, depending on the nature of the comparison. InStat version 2.01 (GraphPad Software) or StatView (version 5) softwares were used. For tail-cuff BP and heart rate measurements, grouping factors were taken into account by using the multiple comparisons procedure. A P value of 0.05 or less was considered significant.

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