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Cardiac phenotype and angiotensin II levels in AT_{1a}, AT_{1b}, and AT_2 receptor single, double, and triple knockouts

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Aims Our aim was to determine the contribution of the three angiotensin (Ang) II receptor subtypes (AT_{1a}, AT_{1b}, AT_2) to coronary responsiveness, cardiac histopathology, and tissue Ang II levels using mice deficient for one, two, or all three Ang II receptors.

Methods and results Hearts of knockout mice and their wild-type controls were collected for histochemistry or perfused according to Langendorff, and kidneys were removed to measure tissue Ang II. Ang II dose-dependently decreased coronary flow (CF) and left ventricular systolic pressure (LVSP), and these effects were absent in all genotypes deficient for AT_{1a}, independently of AT_{1b} and AT_2. The deletion of Ang II receptors had an effect neither on the morphology of medium-sized vessels in the heart nor on the development of fibrosis. However, the lack of both AT_1 subtypes was associated with atrophic changes in the myocardium, a reduced CF and a reduced LVSP. AT_{1a} deletion alone, independently of the presence or absence of AT_{1b} and/or AT_2, reduced renal Ang II by 50% despite a five-fold rise of plasma Ang II. AT_{1b} deletion, on top of AT_{1a} deletion (but not alone), further decreased tissue Ang II, while increasing plasma Ang II. In mice deficient for all three Ang II receptors, renal Ang II was located only extracellularly.

Conclusion The lack of both AT_1 subtypes led to a baseline reduction of CF and LVSP, and the effects of Ang II on CF and LVSP were found to be exclusively mediated via AT_{1a}. The lack of AT_{1a} or AT_{1b} does not influence the development or maintenance of normal cardiac morphology, whereas deficiency for both receptors led to atrophic changes in the heart. Renal Ang II levels largely depend on AT_1 binding of extracellularly generated Ang II, and in the absence of all three Ang II receptors, renal Ang II is only located extracellularly.

Keywords Angiotensin II • G protein-coupled receptors • Genetically modified animals

1. Introduction

The biological actions of angiotensin (Ang) II are mediated via Ang II type 1 (AT_1) and Ang II type 2 (AT_2) receptors. In rodents, two subtypes of AT_1 have been identified: AT_{1a} and AT_{1b}[1] which share 94% sequence homology[2] and have similar ligand binding affinities and signal transduction properties. To date, there are no pharmacological antagonists that discriminate between AT_{1a} and AT_{1b}, and the function of AT_2 is still only partly understood.

To get more insight in the function of the three Ang II receptors, we generated mice which are deficient for either one, two, or all three Ang II receptors.[3] AT_2 deletion increased baseline mean arterial

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pressure (MAP), whereas mice lacking AT_{1a} were hypotensive and displayed a reduced heart weight/body weight (HW/BW) ratio. Blood pressure and HW/BW ratio dropped further in mice lacking both AT_1 subtypes. AT_{1a} deletion impaired the *in vivo* pressor response to Ang II bolus injections, whereas deficiency for AT_{1b} and/or AT_2 had no impact. However, the additional lack of AT_{1b} in AT_{1a}-deficient mice further impaired the vasoconstrictive capacity of Ang II. Ang II failed to alter MAP in mice lacking all three Ang II receptors (triple knockouts), indicating that no other receptors than the AT_{1a}, AT_{1b}, and/or AT_2 mediate the pressor effects of Ang II.

In the present study, we set out to quantify, in the above-described knockout mice, the contribution of the three Ang II receptors to cardiac haemodynamics *in vitro*, using the Langendorff heart preparation. Given the reduced heart size in AT_{1a} knockout mice, we also quantified brain natriuretic peptide (BNP) expression and studied morphological changes such as fibrosis and remodelling. Finally, we measured tissue Ang II levels in these mice to verify earlier findings by us and others showing that tissue Ang II is AT_1-bound.[4–8]

2. Methods

2.1 Animals

Males (aged ~5 months) of all eight possible genotypes ($AT_{1a}/AT_{1b}/AT_2$: +/+//+/+//+/+, −/−//+/+//+/+, +/+//−/−//+/+, +/+//+/+//−/−, +/+//−/−//−/−, −/−//+/+//−/−, −/−//−/−//+/+, −/−//−/−//−/−) of the recently described Ang II receptor deficient mice[3] were used in this study. In addition, age-matched animals lacking angiotensinogen (Agt)[9] were used for histological comparison. Experiments on adult mice were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), the regulations of the Animal Care Committee of the Erasmus MC, and the Federal Law on the Use of Experimental Animals in Germany and were approved by the local authorities (Landesamt für Gesundheit und Soziales des Landes Berlin).

2.2 Langendorff heart preparation

Mice were killed by cervical dislocation. The heart was rapidly excised and placed in ice-cold modified Krebs-Henseleit (KH) buffer (composition in mmol/L: NaCl 118, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2,$CaCl_2$ 1.2, D-glucose 11, $NaHCO_3$ 25, pyruvic acid 2), gassed with 95% O_2 and 5% CO_2.[10–12] The aorta was immediately cannulated with a 19G needle (with a small circumferential grove close to the blunt tip) and perfused with gassed KH buffer according to Langendorff at a constant perfusion pressure of 80 mmHg.[13] Two needle electrodes were placed at the right atrium and the hearts were paced at ~600 b.p.m. (10 Hz, 4 ms duration, 4 V) using a Grass stimulator (Grass Instruments Co., Quincy, MA, USA). Left ventricular systolic pressure (LVSP) was measured with a water-filled balloon (made of domestic food wrap) connected to a disposable pressure transducer (Braun, Melsungen, Germany). The left atrium was removed, and the balloon was inserted into the left ventricle.[13,14] Left ventricular end-diastolic pressure was set at 3–5 mmHg by adjusting the balloon volume. Coronary flow (CF) was measured with a flow probe (Transonic systems, Ithaca, NY, USA). After a stabilization period of 10–15 min, baseline values of CF and LVSP were obtained. Next, bolus injections (100 μL) of modified KH buffer were applied three times to determine injection-induced changes in CF and LVSP. Subsequently, bolus injections (100 μL) of Ang II (concentration range 0.1 nmol/L to 0.1 mmol/L) were applied.

Table 1 Baseline coronary flow (CF) and left ventricular pressure (LVSP) according to genotype

Genotype	+/+//+/+//+/+	+/+//−/−//+/+	+/+//+/+//−/−	+/+//−/−//−/−	−/−//+/+//−/−	−/−//−/−//+/+	−/−//−/−//−/−
n	8	4	6	7	5	7	4	4
CF (mL/min)	2.5 ± 0.2	3.0 ± 0.3	2.8 ± 0.1	3.1 ± 0.1	2.7 ± 0.1	3.0 ± 0.2	2.4 ± 0.3	2.5 ± 0.3
LVSP (mmHg)	98.5 ± 3.7	98.2 ± 6.3	96.5 ± 3.5	98.9 ± 8.3	114.3 ± 6.8	112.6 ± 7.1	86.0 ± 6.2	72.3 ± 6.7

Data are mean ± SEM.

2.3 RNA isolation and RNase protection assay

Total RNA was isolated from heart using the TRIzol reagent (Invitrogen GmbH, Karlsruhe, Germany) as described earlier.[15] Mouse BNP mRNA expression was identified by RPA using the Ambion RPA II kit (Ambion Europe Ltd, Huntingdon, UK) as described elsewhere.[15] In brief, SP6-RNA polymerase transcribed a radioactive antisense probe complementary to BNP mRNA (290 bp).[16] RNA complementary to 127 nucleotides of rL32 mRNA was used as a positive control.[17,18] Thirty microgram of each RNA sample was hybridized with of the radiolabelled antisense probes in the same reaction. The hybridized fragments protected from RNase A + T1 digestion were separated by electrophoresis and analyzed using a FUJIX BAS 2000 Phospho-Imager system (Raytest GmbH, Straubenhardt, Germany). Quantitative analyses were performed by measuring the intensity of the target bands normalized by the intensity of rL32.

2.4 Histology

Hearts were isolated and fixed in 4% buffered formalin and processed according to standard protocols. In brief, the hearts were embedded in paraffin and sectioned at 2 μm. Slides were deparaffinized and hydrated. Sections of hearts were stained for histology with haematoxylin-eosin (HE) solution, periodic acid-Schiff (PAS) solution, or van Gieson solution as described previously.[18,19] Tissue sections ($n \geq 4$) were evaluated in four planes for morphological changes and connective tissue production.

2.5 Ang II levels in kidney

Kidneys were removed from the abdomen, rapidly frozen in liquid nitrogen, and stored at −80°C until processing. Ang II was determined by radioimmunoassay following SepPak extraction and high-performance liquid chromatography separation as described before.[20]

2.6 Data analysis

CF and LVSP data were recorded and digitalized using WinDaq waveform recording software (Dataq Instruments, Akron, OH, USA). After a manual selection of the desired signals pre- and post-injection, data were analysed using Matlab (Mathworks, Inc., Natick, MA, USA). Six consecutive beats were selected for the determination of CF and LVSP. Results are represented as mean ± SEM or geometric mean and range. Concentration–response curves were analysed as described,[21] using Graph Pad Prism 3.01 (Graph Pad Software, Inc., San Diego, CA, USA), to obtain pEC_{50} ($-^{10}\log EC_{50}$) values. The pEC_{50} values refer to the agonist concentration in the injection fluid and do not reflect the actual concentrations seen by the receptor. Statistical analysis was performed using the SPSS 11.0 statistical package (SPSS, Inc., Chicago, IL, USA). Multiple regression analysis was conducted to determine the contribution of the three receptors as independent variables and, in case one of the receptors did not exert an independent effect, their interaction. $P < 0.05$ was considered significant.

3. Results

3.1 Langendorff heart studies

Table 1 shows the baseline CF and LVSP values in the eight genotypes. Regression analysis revealed that the deletion of individual Ang II receptor subtypes did not affect baseline CF. However, combined deletion of AT_{1a} and AT_{1b}, independently of the presence or absence of AT_2, lowered baseline CF ($P = 0.020$; *Figure 1*, left panel). Although baseline LVSP was unaffected by individual AT_{1a} or AT_2 deletion, it was lower in hearts lacking AT_{1b}, independently of the presence of AT_{1a} or AT_2 ($P = 0.005$; *Figure 1*, middle panel). In addition, AT_{1a} deletion on top of AT_{1b} deletion further decreased baseline LVSP ($P = 0.003$; *Figure 1*, right panel).

In hearts of wild-type mice, bolus injections of Ang II dose-dependently decreased CF and LVSP (*Figure 2*) by maximally 56 ± 5% and 39 ± 4%, respectively (pEC_{50} 7.51 ± 0.17 and 7.35 ± 0.13). Ang II concentrations >1 μmol/L did not result in effects that were larger than those observed at 1 μmol/L, in agreement with the concept of receptor desensitization.[22–24]

Bolus injections of Ang II exerted no effects in hearts of mice deficient for AT_{1a} receptors. Interestingly, no differences were found between hearts of mice lacking AT_{1a} alone and hearts of mice deficient for AT_{1b} and/or AT_2 on top of AT_{1a} deficiency. The effects of Ang II bolus injections in hearts of mice lacking AT_{1b} and/or AT_2 were identical to those in wild-type mice. This suggests that the cardiac effects of Ang II are exclusively AT_{1a} mediated.

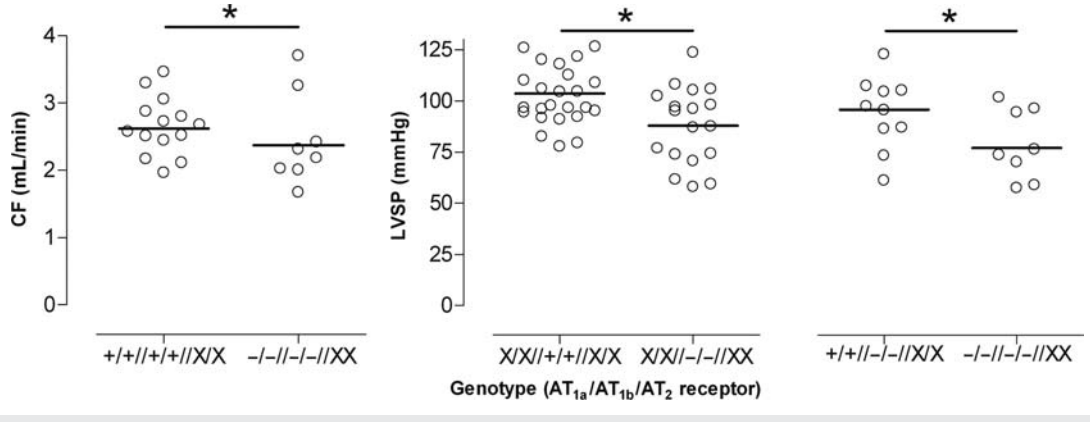

Figure 1 Baseline coronary flow (CF) following deletion of AT_{1a} and AT_{1b}, independently of the presence or absence of AT_2 (left panel), baseline left ventricular systolic pressure (LVSP) following deletion of AT_{1b}, independently of the presence of AT_{1a} or AT_2 (middle panel), and baseline LVSP following deletion of AT_{1a} on top of AT_{1b}, independently of the presence of AT_2 (right panel). *$P < 0.05$; data ($n = 8–26$) are represented as scatter dot plot. The horizontal bar represents the mean. The use of 'X' implies that for that specific receptor, both +/+ and −/− animals were included, i.e. that the comparison occurred independently of the presence or absence of that receptor.

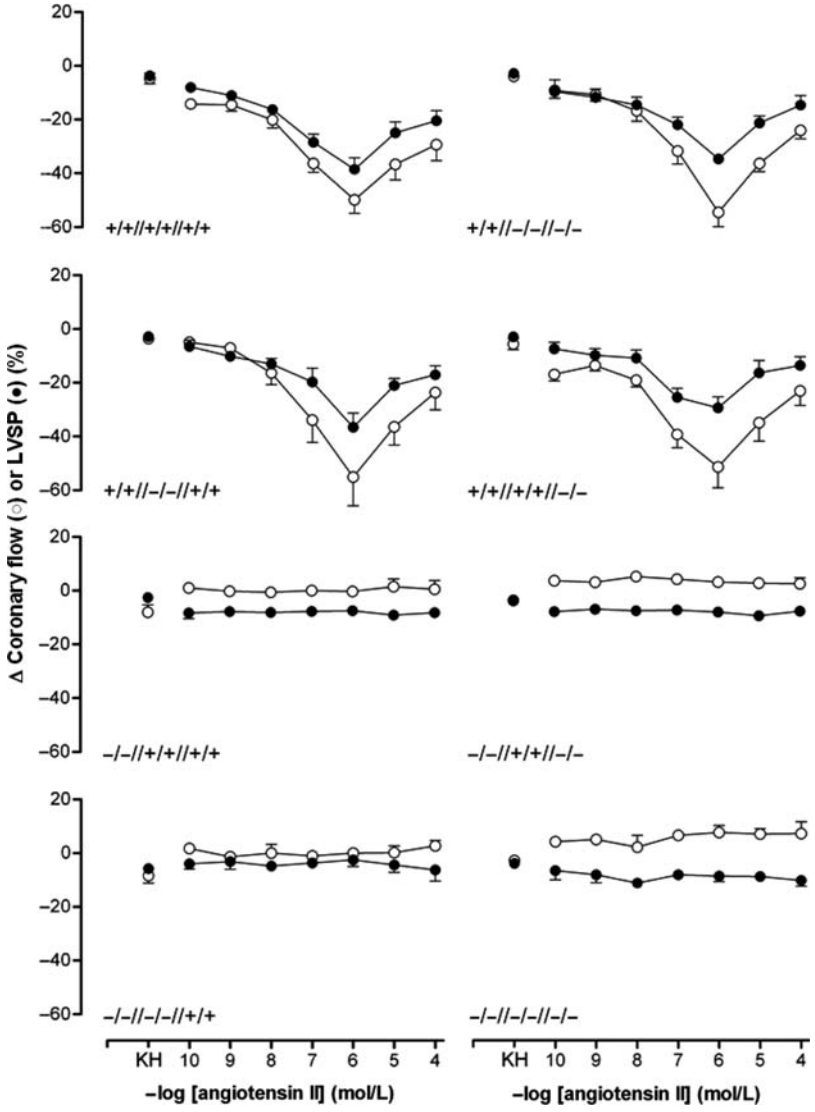

Figure 2 Changes in coronary flow (CF; open symbols) and left ventricular systolic pressure (LVSP; closed symbols) after a bolus injection of Ang II in the Langendorff heart according to genotype. Data (mean + SEM of 4–8 experiments) represent percentage change from baseline. KH, bolus injection of Krebs-Henseleit buffer. The *x*-axis displays the Ang II concentration in the injection fluid.

3.2 Cardiac histopathological examination and expression of BNP

We investigated the impact of the Ang II receptors on morphological changes in medium-sized vessels in the heart using PAS-stained sections. Deficiency for one, two, or all three Ang II receptor subtypes did not influence the morphology of medium-sized arteries in the heart (*Figure 3*). To investigate the impact of lack in Ang II receptor expression on fibroblast quantities, we determined the basal production of connective tissue by van Gieson staining. In none of the eight possible receptor combinations, a change in basal connective tissue production was detected (*Figure 4*).

As we have reported earlier, all genotypes lacking the AT_{1a} receptor showed a significantly reduced HW/BW ratio.[3] Interestingly, there was oligo- to multi-focal atrophy of cardiac muscle evident in animals lacking both AT_1 subtypes (−/−//−/−//+/+ and −/−//−/−//−/−) and Agt-deficient mice, whereas animals deficient for none or only one of the AT_1 subtypes showed no structural changes within the heart (*Figure 5*). Atrophic muscle fibres revealed a more pronounced, dense, reticular network, with partial collapse of the reticulin fibres. Atrophic muscle fibres had an angular outline, and widened partially enlarged interstitial spaces.

BNP is a marker for heart failure. Thus, we tested whether the deficiency of one of the receptors and/or the structural changes have an impact on BNP expression in all eight groups. None of the eight genotypes showed increased BNP expression (data not shown).

3.3 Ang II levels in plasma and kidney

As the hearts had already been used for *ex vivo* haemodynamic studies, these measurements were performed in kidneys. Kidneys have much higher Ang II levels than the heart,[25] and changes in renal Ang II content exactly parallel changes in the Ang II content of other tissues (including the heart).[25–27] In other words, the renal Ang II

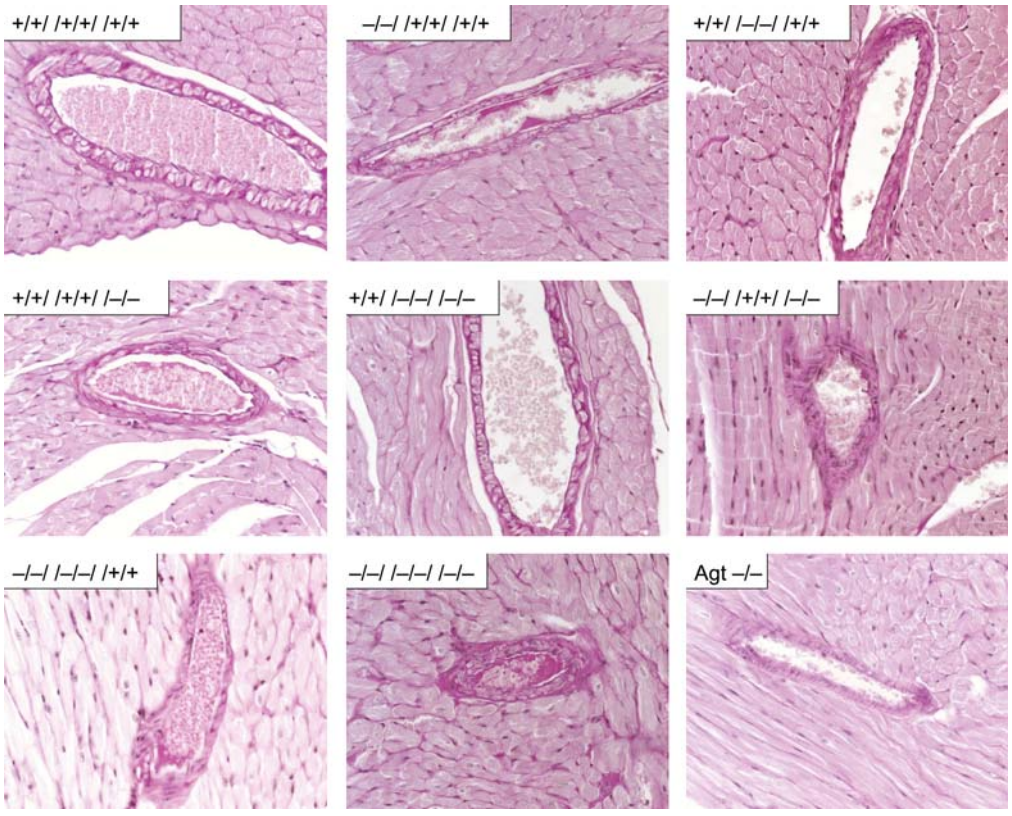

Figure 3 Medium-sized arteries in hearts of wild-type (+/+//+/+//+/+), AT_{1a}- (−/−//+/+//+/+), AT_{1b}- (+/+//−/−//+/+), and AT_2- (+/+//+/+//−/−) single-knockout mice, mice exclusively expressing AT_{1a} (+/+//−/−//−/−), AT_{1b} (−/−//+/+//−/−), or AT_2 (−/−//−/−//+/+), triple-knockout mice (−/−//−/−//−/−), and angiotensinogen-deficient (Agt −/−) mice do not show genotype-related morphological alterations (PAS-stained; magnification: 400-fold; representative sections for all groups from $n \geq 4$ sections).

levels can be used as a reflection of what happens at the tissue level in general. Renal Ang II levels were compared with the plasma Ang II levels (in the same animals) reported previously by our group.[3] For the sake of clarity, *Figure 6* shows the geometric mean and range of the Ang II levels in both kidney (A) and plasma (B). Regression analysis revealed that AT_{1a} deletion alone, independently of the presence or absence of the AT_{1b} and/or AT_2, diminished renal Ang II by ≈50% ($P = 0.002$; *Figure 7A*, left panel) and increased plasma Ang II ≈5-fold ($P < 0.0001$; *Figure 7B*, left panel).

AT_{1b} deletion alone did not affect plasma and renal Ang II (*Figure 6*). However, on top of AT_{1a} deletion, independently of the presence or absence of the AT_2, AT_{1b} deletion further diminished renal Ang II by a factor of 4 ($P < 0.0001$; *Figure 7A*, middle panel) and doubled plasma Ang II ($P = 0.003$; *Figure 7B*, middle panel).

AT_2 deletion alone did not affect plasma and renal Ang II. However, on top of AT_{1a} deletion, independently of the presence or absence of the AT_{1b}, AT_2 deletion reduced plasma (but not renal) Ang II ($P = 0.018$; *Figure 7A* and *B*, right panels).

We have previously shown that tissue Ang II is protected against metabolism by binding to AT_1 and subsequent internalization.[7] To verify this concept, kidneys of two mice lacking all three Ang II receptors were kept at room temperature for 30 min before freezing them in liquid nitrogen. Ang II levels in these kidneys were 2 and 8 fmol/g, whereas the (geometric) mean Ang II level in the kidneys of AT_{1a}/AT_{1b}/AT_2 triple knockout mice that had been frozen immediately was 35 fmol/g.

4. Discussion

We have previously shown that AT_{1a} deletion impaired the *in vivo* pressor response to Ang II bolus injections, whereas deficiency for AT_{1b} and/or AT_2 had impact only on top of AT_{1a} deletion.[3] The importance of AT_{1a} is also apparent in our current studies using the mouse heart Langendorff model. Deletion of one, two, or all three Ang II receptors did not affect baseline CF or LVSP in comparison to wild-type. The dose-dependent decrease of both CF and LVSP in response to Ang II was similar in all genotypes expressing AT_{1a} independently of the presence or absence of AT_{1b} and/or AT_2. These effects were completely abolished in all genotypes deficient for the AT_{1a}. Thus, the AT_{1a} exclusively mediates the Ang II-induced negative inotropy and vasoconstriction in the mouse heart, and AT_{1b} does not exert such effects, possibly because it is not expressed in the heart in sufficient amounts. Whether the negative inotropic and vasoconstrictor effects of Ang II occurred independently of each other could not be determined in the present study. Negative inotropic effects of Ang II in the mouse heart have been described before,[28,29] and contrast with the positive inotropic effects of Ang II in human trabeculae.[30] Yet, it is also possible that the decrease in LVSP simply is the consequence of the Ang II-induced decrease in CF, i.e. that Ang II has no direct effect on the mouse cardiomyocyte.

Unexpectedly, absence of the AT_2 did not enhance the AT_1-mediated effects in the heart. This contrasts with other studies in the heart where

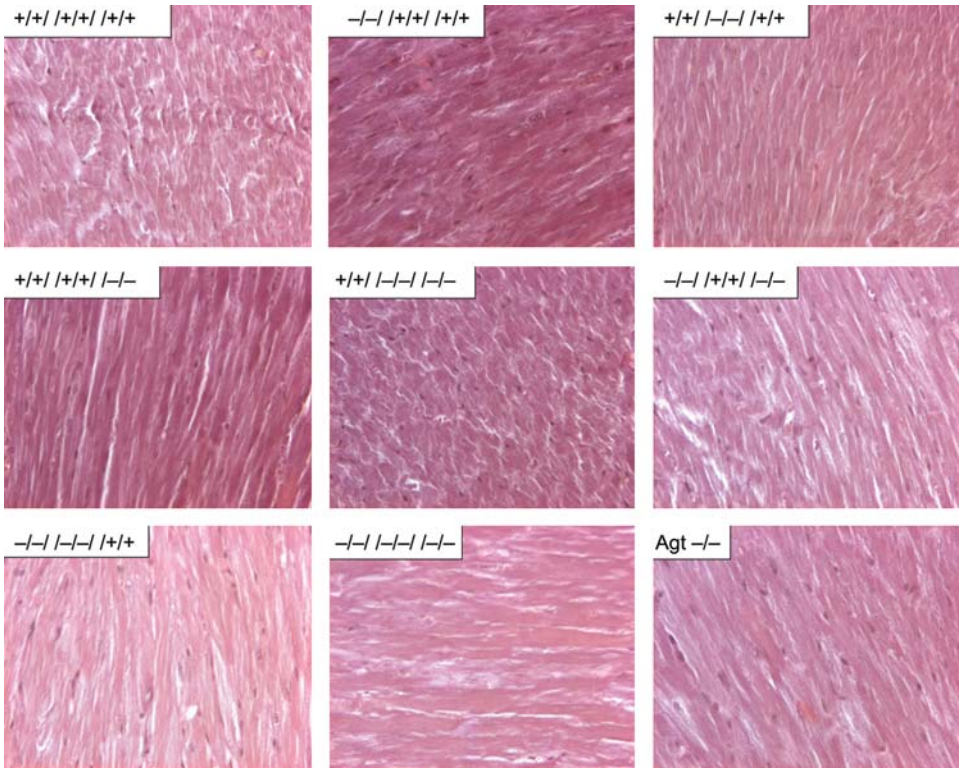

Figure 4 Sections of hearts from wild-type (+/+//+/+//+/+), AT_{1a}- (−/−//+/+//+/+), AT_{1b}- (+/+//−/−//+/+), and AT_2- (+/+//+/+//−/−) receptor single-knockout mice, mice exclusively expressing AT_{1a} (+/+//−/−//−/−), AT_{1b} (−/−//+/+//−/−), or AT_2 (−/−//−/−//+/+) receptors, triple-knockout mice (−/−//−/−//−/−), and angiotensinogen-deficient (Agt −/−) mice do not show changes in basal connective tissue production (van Gieson-stained; magnification: 400-fold; representative sections for all groups from $n \geq 4$ sections).

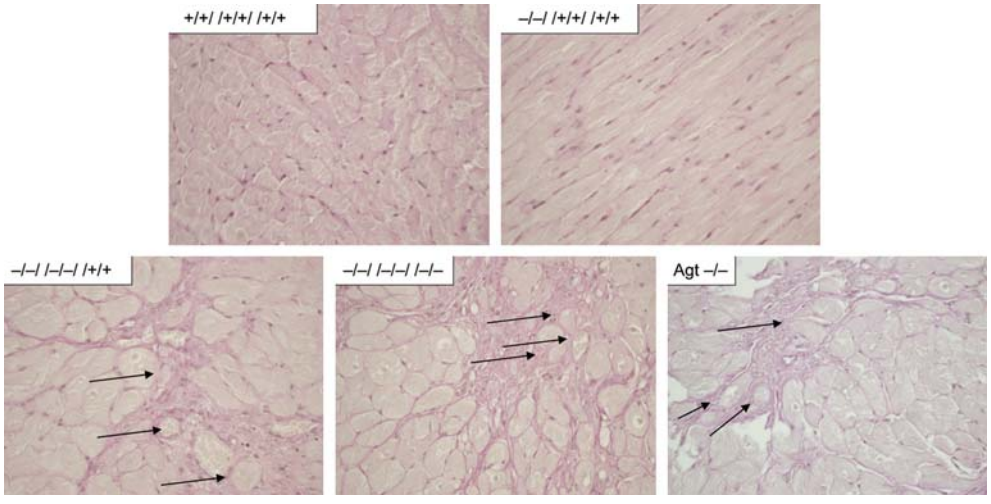

Figure 5 Oligo- to multi-focal atrophy of cardiac muscle is evident in animals lacking both AT_1 subtypes (−/−//−/−//+/+ and −/−//−/−//−/−) and angiotensinogen-deficient (Agt −/−) mice, whereas animals lacking none or only one AT_1-subtype (exemplarily shown +/+//+/+//+/+ and −/−//+/+//+/+). Arrows indicate dense reticular network, partially collapsed, with individualized muscle fibres, and occasional angular outline, as well as loss of muscle fibres (HE-stained; magnification: 400-fold representative sections for all groups from $n \geq 4$ sections).

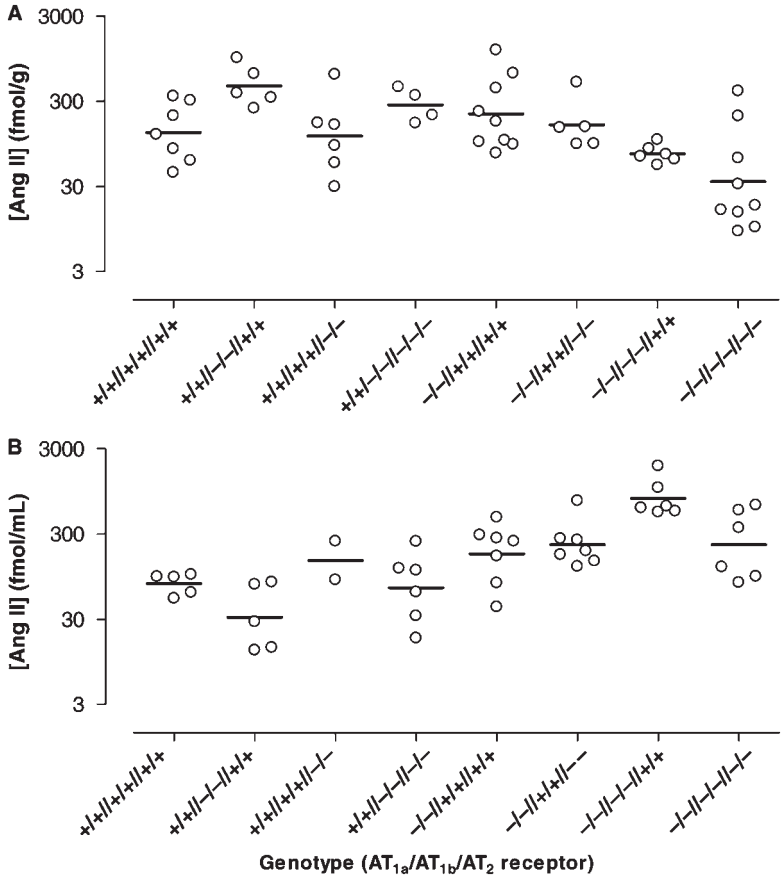

Figure 6 Ang II levels in kidney (*A*) and blood plasma (*B*) of wild-type (+/+//+/+//+/+), AT_{1a}- (−/−//+/+//+/+), AT_{1b}- (+/+//−/−//+/+), and AT_2-single-knockout mice, mice exclusively expressing AT_{1a} (+/+//−/−//−/−), AT_{1b} (−/−//+/+//−/−), or AT_2 (−/−//−/−//+/+), and triple-knockout mice (−/−//−/−//−/−). Data ($n = 2–9$) are represented as scatter dot plot. The horizontal bar represents the geometric mean. Plasma levels were redrawn from Gembardt *et al.*[3]

AT_2 blockade potentiated the effects of Ang II.[12,31–33] On the basis of these enhanced effects, it has been concluded that AT_2 counteract the AT_1-mediated effects. An explanation for the absence of such enhanced cardiac effects in the AT_2 knockout mice in the present study could be that chronic absence (unlike acute blockade) of AT_2 affects AT_{1a} signalling modifying pathways. Alternatively, life-long absence of AT_2 may have resulted in structural changes. However, histological examination did not reveal such alterations, and baseline CF and LVSP were identical in all genotypes. Moreover, the vascular and cardiac effects of other agonists (phenylephrine and endothelin-1) in these mice are unaltered.[3] Thus, the discrepancy between pharmacological blockade and the AT_2 knockout related to specific parameters could be also a result of ectopic effects of the AT_2 blocker as significant ectopic effects have been described for a variety of AT_1 blockers.[34,35] The AT_2 blocker used in these studies, PD123319, has been originally described as an AT_2 agonist.[36] Taken this in consideration, the genetically receptor-deficient mice are a superior experimental model compared with the pharmacological blockade of AT_2.

Furthermore, we also did not observe direct AT_2-mediated effects in AT_1 knockout mice. This is in line with our previous inability to observe cardiac AT_2-mediated effects during AT_1 blockade in wild-type mice.[12] Apparently therefore, AT_2-mediated effects in the mouse heart can only be demonstrated during simultaneous AT_1 stimulation, i.e. they occur in conjunction with AT_{1a} activation. However, our findings also show that this concept does not account for all tissues or Ang II-mediated effects. Our data on plasma Ang II levels implicate AT_2-mediated effects independent of AT_1, because AT_2 deficiency led to lower Ang II concentrations in plasma of AT_1-deficient animals.

Recently, we reported reduced heart weights for mice lacking AT_{1a} in all combinations, in AT_2-deficient mice, and mice lacking Agt.[3] In this study, we found a similar, genotype-specific atrophy in the myocardium of AT_{1a}/AT_{1b} double-, $AT_{1a}/AT_{1b}/AT_2$ triple-, and Agt-deficient mice. Interestingly, mice only deficient for AT_{1a} and not for AT_{1b} (−/−//+/+//+/+ and −/−//+/+//−/−) did not show the described atrophic changes. Despite the low AT_{1b} expression in the heart, our data suggest a rescue of AT_{1a} function by AT_{1b} in the maintenance of normal cardiac morphology, whereas AT_{1b} cannot compensate for the lack of growth stimulation in the heart due to the absence of AT_{1a}. The reduction in baseline CF and LVSP in mice deficient for both AT_{1a} and AT_{1b} may be the consequence of these atrophic changes. Thus, atrophy alone cannot explain the reduced heart weight in the five genotypes where lower organ weight has been observed, because mice deficient for AT_{1a} and/or AT_2 (−/−//+/+//+/+, +/+//+/+//−/−, and −/−//+/+//−/−) do not show the atrophic changes seen in mice lacking both AT_1

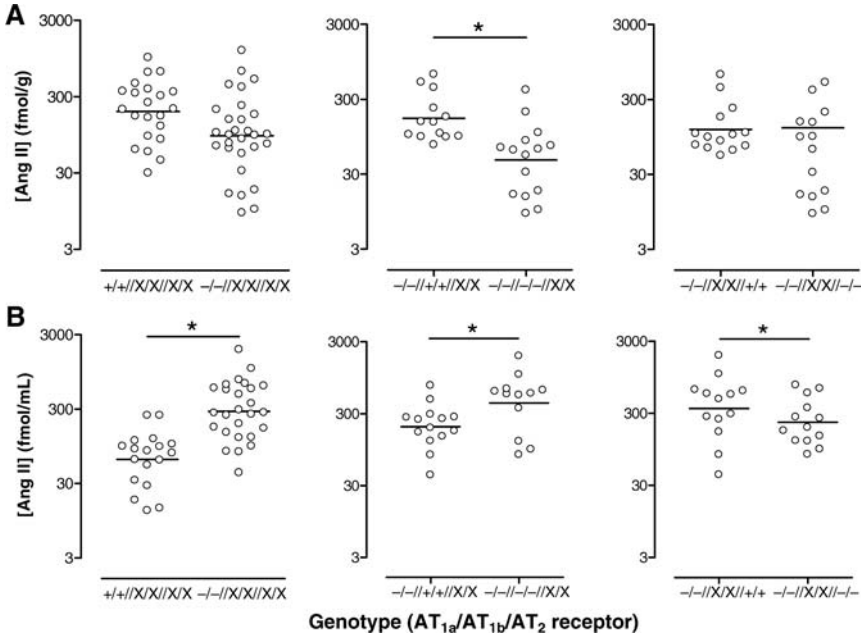

Figure 7 Ang II levels in kidney (*A*) and blood plasma (*B*) following deletion of AT_{1a}, independently of the presence or absence of AT_{1b} or AT_2 (left panels), deletion of AT_{1b} on top of AT_{1a} deletion, independently of the presence of AT_2 (middle panels), or deletion of AT_2 on top of AT_{1a} deletion, independently of the presence of AT_{1b} (right panels). $^*P < 0.05$; Data ($n = 12-29$) are represented as scatter dot plot. The horizontal bar represents the geometric mean. The use of 'X' implies that for that specific receptor, both +/+ and −/− animals were included, i.e. that the comparison occurred independently of the presence or absence of that receptor. Plasma levels were obtained from Gembardt *et al.*[3]

subtypes. Therefore, further experiments have to investigate both phenomena; the reduced heart weight and the atrophy in the heart of mice with lacking AT_{1a}/AT_{1b} stimulation.

Finally, the present study yielded important information on the regulation of tissue Ang II levels. Normally, circulating Ang II is sequestered by multiple tissues via AT_1-mediated endocytosis.[7] Yet, the majority of tissue Ang II is formed at tissue sites.[37,38] Studies with ^{125}I-labelled Ang I have revealed that this production occurs extracellularly and is followed by rapid AT_1-mediated endocytosis.[4–8] Therefore, despite its extracellular synthesis, e.g. in the interstitial fluid compartment or on the cell surface, the majority of tissue Ang II is located intracellularly.[8] Its levels are particularly high in endosomes.[39] The intracellular accumulation of Ang II may either facilitate its activation of nuclear receptors[40,41] and/or results in its destruction.[7] The half-life of AT_1-bound tissue Ang II is 20–30 times longer than that of extracellular Ang II,[7] thereby explaining, at least in part, why tissue Ang II levels far exceed plasma Ang II levels. Clearly, receptor-binding protects Ang II against rapid metabolism by angiotensinases.

As AT_2 does not internalize after ligand binding,[42,43] the intracellular accumulation of Ang II exclusively depends on AT_1. The current study now shows that both AT_1 subtypes contribute to this accumulation and that the contribution of the AT_{1a} exceeds that of the AT_{1b}. Making use of single AT_{1a} knockout mice, a similar conclusion was already drawn by Li and Zhuo.[44]

Not having AT_{1a} caused a five-fold rise in plasma Ang II. Despite this rise in extracellular Ang II, the renal tissue level of Ang II decreased by 50%. Not having AT_{1b} does not have an effect by itself on either plasma or tissue Ang II, thereby demonstrating that AT_{1a} can make up for the consequences of the deletion of AT_{1b}. However, missing AT_{1b} on top of AT_{1a} deletion further increased plasma Ang II (by a factor of 2) and lowered tissue Ang II by a factor of 4. Therefore, in mice not expressing AT_1, the tissue/plasma Ang II concentration ratio is far below that in wild-type mice. Additional AT_2 deletion did not alter renal Ang II, in agreement with the above concept of non-internalizing AT_2.

Renal Ang II levels in the absence of its three receptors are not zero. To verify whether the measured Ang II is putatively extracellular in $AT_{1a}/AT_{1b}/AT_2$-triple knockout mice, we determined the Ang II content of kidneys that were kept at room temperature for 30 min. Normally, over this time period, the level of AT_1-bound Ang II does not change,[7] whereas the level of extracellular Ang II rapidly decreases.[6] Indeed, tissue Ang II levels in mice deficient for all three Ang II receptors following this procedure decreased by more than 70%, confirming the rapid metabolism of non-receptor bound, extracellular Ang II by angiotensinases. Furthermore, given that the extracellular fluid content of renal tissue consists of blood plasma and interstitial fluid (accounting for ≈5% and ≈10% of tissue weight, respectively), and assuming that the renal interstitial Ang II levels resemble those in blood (≈320 fmol/mL in triple knockout mice), it can be estimated that the renal tissue Ang II levels in triple knockout mice should be in the order of ≈50 fmol/g, if Ang II is limited to the extracellular fluid compartment. This is within the range of the tissue levels that were found (35 fmol/g), thereby again confirming that tissue Ang II in triple knockout mice is extracellular. Consequently, the experimental data in our triple knockout mice clearly argue against the formerly expressed hypothesis of intracellular Ang II generation.[45]

On the basis of our new findings using animals with different combinations of Ang II receptor deficiencies, we can draw the following conclusions: (i) the lack of both AT_1-subtypes led to reduced baseline CF and LVSP, (ii) the coronary constrictor and negative inotropic effects of Ang II are exclusively mediated via AT_{1a}, (iii) deficiency of only one AT_1 subtype has no effect on development or maintenance of normal cardiac morphology, whereas the lack of both receptors led to atrophic changes in the heart, (iv) tissue Ang II levels largely depend on AT_1-mediated endocytosis of extracellular Ang II; (v) in the absence of the three Ang II receptors, tissue Ang II is only located extracellularly.

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