The intronic angiotensin II type 2 receptor gene polymorphism 1675 G/A modulates receptor protein expression but not mRNA splicing

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Abstract

The X-linked angiotensin II type 2 receptor (AT2) is supposed to be involved in cardiovascular disorders. Two studies associated the A allele of the AT2 gene polymorphism (PM) 1675 G/A with left ventricular hypertrophy in men and coronary ischemia in women. Since the PM is located in the short intron 1 of the AT2 gene within a sequence motif similar to the splice branch site consensus, we tested whether it might affect pre-mRNA splicing and/or modulate AT2 receptor expression.

We first analyzed the AT2 mRNA splice pattern by RT-PCR in myocardial samples from 12 explanted human hearts and compared it with the respective genotypes. All 12 patients, 10 hemizygous males (7 A, 3 G allele carriers) and 2 homozygous females (2 G/G allele carriers), exhibited the same myocardial AT2 splice pattern with a relative abundance of transcript exon 1/2/3 compared with exon 1/3. Next, AT2 minigene constructs were cloned from both alleles, comprising the core promoter and the complete transcribed region up to the translation start codon, upstream of a luciferase reporter gene. These constructs were transfected into human (HT1080) and rat (PC12W) cell lines and rat vascular smooth muscle cells, and luciferase activities were assessed as well as the splice patterns of the chimaeric AT2/luciferase mRNAs. In all transfected cell types the mRNA expressed from the AT2 constructs was spliced like the endogenous myocardial AT2 mRNA. However, luciferase activities driven by the G allele construct were significantly higher than those expressed from the A allele.

Taken together these data indicate that individuals carrying the G allele may express higher levels of AT2 receptor protein which may be protective during the development of ventricular hypertrophy and coronary ischemia.
Keywords: angiotensin II, receptor, single nucleotide polymorphism, RNA splicing, gene expression
**Introduction**

The role of the angiotensin II type 2 receptor (AT2) in the development of cardiovascular disease is still a matter of debate. AT2 receptor stimulation seems to counteract cell proliferation, cardiomyocyte hypertrophy and fibrosis induced by AT1 stimulation and does probably play a role in human cardiovascular disease (1-8). Structurally, both receptor subtypes belong to the class of seven-transmembrane receptors, but they are only distantly related and coupled to completely different signaling pathways. It has been suggested that AT2 may counteract AT1 effects by activation of protein phosphatases that downregulate protein kinase activities induced by AT1 or tyrosine kinase receptor stimulation (for review see 10;11) by ligand-independent direct interaction with AT1 (12;13), by competition for angiotensin II and / or downregulation of AT1 expression (13-15). Alternatively, antagonism may just appear at the functional level which does not require expression of both receptors by the same cell type: several studies showed that stimulation of vascular, mainly endothelial, AT2 receptors may increase bradykinin and NO release which induces vasodilation and reduces proliferation (6;13;16;17). This mechanism probably underlies the blood pressure lowering AT2 effect which was first deduced by disruption of the AT2 gene in mice (18;19). Results obtained from AT2 knock outs in experimental cardiac remodeling induced by aortic banding or chronic Ang II infusion seem to depend on the genetic background demonstrating an increase of myocyte hypertrophy and cardiac fibrosis in the presence of AT2 in some studies (20;21), and reduction of coronary artery remodeling and perivascular fibrosis in another (22). In experimental myocardial infarction in mice the presence of AT2 receptors improved cardiac function and reduced mortality (23;24). Likewise, overexpression of AT2 in cardiomyocytes preserved cardiac function during post-infarct remodeling (25) and reduced perivascular fibrosis of intramuscular coronary arteries after Ang II infusion (26). Since AT2
is only expressed at low levels in the healthy adult vasculature and heart, particularly in rodents, but is upregulated in vascular and myocardial remodeling (27-29) AT2 effects on cardiovascular structure and function may only become detectable under pathological conditions and/or AT1 blockade.

The association of an intron 1 AT2 polymorphism, designated as 1675 G/A or –1332G/A based on its location relative to transcription or translation start, with myocardial structure and cardiac disease has been investigated in several studies (30-34). The A-allele was associated with higher left ventricular mass in young male Bavarian untreated hypertensives and with a greater cardiovascular risk in 2579 healthy UK men (33). In contrast, the G allele was found to be associated with higher LV mass in 125 male UK patients with systemic hypertension (34). In the European Project on Genes in Hypertension (EpoGH), different associations of the AT2 polymorphism with LV mass were found depending on sodium excretion in males but not in females (32). In the Glasgow Heart Scan Old study the A-allele was associated with left ventricular hypertrophy in males and recurrent coronary ischemia and myocardial infarction in elderly females (31). Until now it could only be speculated which of the AT2 alleles may be associated with a higher AT2 expression, and the molecular mechanisms underlying these putative differences in receptor expression have been obscure.

Since it was suggested that the intronic AT2 1675 G/A PM might affect pre-mRNA splicing (35), the aim of the present work was to verify this hypothesis or to find alternative explanations for the observed phenotypes. For this purpose we used myocardial samples from explanted human hearts and transgenic cell cultures models.
Materials and Methods

Analysis of the effect of the AT2 1675 genotype on the myocardial AT2 splice pattern

In a previous study (36) we analyzed by RT PCR the myocardial AT2 splice pattern of 14 explanted failing hearts from patients who had undergone orthotopic heart transplantation at the German Heart Institute (see table 1 for patient data). Twelve of these patients were now genotyped using single stranded conformation polymorphism (SSCP) PCR and nucleotide sequencing of the PCR products as described previously (30;37).

Analysis of the effect of the AT2 PM in transfected human and rodent cells

*Cell culture and reagents:* Cell culture reagents were purchased from Gibco BRL Life Technologies (Karlsruhe, Germany) and PAA (Cölbe, Germany). Human fibrosarcoma HT1080 cells were from DSMZ (German Collection of Microorganisms and Cell Cultures; Braunschweig, Germany). Rat vascular smooth muscle cells were prepared from adult rat aorta as described previously (38) and used until passage 10. Both cells types were cultured in DMEM, supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin). Rat PC12W cells were a kind gift from Prof. S. Bottari (INSERM, Grenoble, France) and were cultured in RPMI 1640, 10% horse serum, 5% fetal bovine serum and antibiotics.

*Cloning of AT2 minigene/luciferase constructs:* The genomic human AT2 sequence from nt 1147 to nt 3003 (according to accession no. U20860; (34) was cloned in the promoterless luciferase reporter gene vector pGL2basic (Promega, Madison, WI, U.S.A.) using generated Sac I and Hind III restriction sites in the primers. The A allele was cloned from a human genomic library as described previously (construct AT2Ex3 in (40)). The G allele was PCR-
amplified from human genomic DNA using a DNA polymerase with proof-reading activity (Combi Pol\textsuperscript{TM}, Invitex, Berlin Germany). The constructs contained 293 bp of the AT2 core promoter (40), and the complete transcribed region of the AT2 gene, including the two introns, up to the translation start codon in exon 3, which was replaced by the luciferase translation start. The identity of the constructs was confirmed by DNA sequencing.

**Transfections:** Five micrograms of the reporter plasmids, 18 µg of pBluescriptSK+ DNA (Stratagene, La Jolla, CA, U.S.A) and two µg of a β-galactosidase expression plasmid (pCMV-β-gal, Stratagene) were transiently introduced into HT1080, PC12W or adult rat vascular smooth muscle cells by electroporation as described previously (36;40). Transfection efficiencies were 35-45% for HT1080 and PC12W cells and 5-10% for rat smooth muscle cells. Four separate plasmid preparations were used for the transfection experiments.

**Analysis of the hAT2 splice pattern in transfected cells:** One day post-transfection cells were harvested and poly A+ RNA was prepared using the Dynabeads mRNA Purification Kit (Dynal, Oslo, Norway) according to the manufacturer’s protocol. One tenth of the RNA was reverse transcribed with Superscript II Reverse Transcriptase (Gibco Life Technologies), and 20% of the resulting cDNA was PCR-amplified in a Perkin Elmer 9600 Thermo Cycler with a primer pair binding to the cDNA of the human AT2 construct (AT2Ex1for: 5´ CAG AAT TCA AAG CAT TCT GCA GCC, AT2Ex3rev 5´CTT AAG CTT GAA ATG TCA GCA GCT CC underlined the generated Hind III restriction site, 30 cycles for HT1080 and PC12W cells, 32 cycles for rat vascular smooth muscle cells), but not to the cDNA of the endogenous rat AT2 expressed in PC12W cells. The expected size of the PCR products was 77 bp for the splice variant exon 1/3 and 136 bp vor variant exon 1/2/3. As a control for equal RNA input and quality, a β actin PCR was performed in parallel on 10% of the cDNA (β actin + 5´-AGGCCGGCTTCGCGGGCGAC; β actin - 5´-CTCGGGAGCCACACGCAGCTC; 245-bp
amplificate, 26 cycles). PCR products were separated on a 2% agarose gel and visualized by ethidiumbromide staining.

**Luciferase reporter gene assays:** For the quantification of AT2 allele-driven luciferase expression, cells were lysed two days post-transfection. Luciferase and β-galactosidase activities were measured by the use of Luciferase Assay Reagent and β Galactosidase Enzyme Assay System, respectively (Promega). Luciferase activities were normalized in reference to β-galactosidase activities. Luciferase activity of the A allele construct were set to 100%. Data are given as mean values of 8 to 10 independent experiments with three replicates per construct±SEM.

**Statistics:** Statistical analysis was performed by Student’s t-test. An alpha level of 0.05 was used to determine significance.
Results

The AT2 gene 1675 G/A polymorphism (PM) is located within a putative branch site consensus motif in the small intron 1 (Fig. 1A and B). Therefore, we examined whether it could modulate pre-mRNA splicing qualitatively or quantitatively and/or affect the level of protein expression.

*The AT2 mRNA splice pattern in explanted human hearts is not modified by the AT2 PM:* We first determined the AT2 genotype of patients who had undergone orthotopic heart transplantation in 1997 and 1998 in the German Heart Institute and whose explanted hearts had been analyzed for the myocardial AT1 and AT2 splice pattern in a previous study (36). In this RT PCR based study we found identical AT2 splice patterns in all patients, irrespective of their clinical diagnosis, stage of disease, sex or cardiac localization of the sample (i.e. atrium or ventricle, Fig. 2). In all patients the splice variant exon 1/2/3 was the most abundant AT2 mRNA (92%) compared with the small splice variant exon 1/3 (8%) (36). The SD were low for the highly abundant splice variant Ex 1/2/3 (92 +/-8%), but were high for the low abundancy variant Ex 1/3 (8+/ -12 %). However the differences between expression of both mRNAs were highly significant. Importantly, irrespective of the genotype the splice variant Ex 1/2/3 could be detected in all samples tested and the smaller transcript Ex 1/3 was only close to the detection limit when total AT2 mRNA expression was low. For the present study 12 of these patients (10 males, 2 females) were genotyped. Seven males were hemizygous for the AT2 A allele and 3 for the G allele; the two female patients were homozygous for the G allele (Table 1). These *ex vivo* data demonstrate that the myocardial AT2 mRNA splice pattern is not detectably affected by the 1675 genotype.

*The AT2 splice pattern is not affected by the PM in cells transiently transfected with minigene constructs of the two alleles:* Since there is no human cell line expressing the AT2
endogenously, and primary cells such as vascular endothelial cells down-regulate it rapidly in cell culture (2). AT2 minigene constructs were used to investigate the effect of the PM in cell cultures. The minigene constructs contained a genomic AT2 fragment of either allele comprising the core promoter, exon 1 and 2, intron 1 and 2, and exon 3 up to the translation start codon upstream of the luciferase coding region (Fig. 3 A). These plasmids were transiently transfected in human HT1080, rat PC12W and rat vascular smooth muscle cells. RT PCR analysis of the overexpressed chimaeric human AT2/luciferase mRNA revealed that all three cell lines expressed the same splice pattern, irrespective of the AT2 genotype, and this pattern was identical to the previously identified myocardial AT2 splice pattern, i.e. mRNA exon 1/2/3 was the predominant variant in HT1080 and PC12W cells (Fig. 3 B and C, respectively) as well as in rat vascular smooth muscle cells (not shown). Plasmids carrying the spliced AT2 cDNAs consisting of exon 1/2/3 and exon 1/3 served as positive controls (Fig. 3 B and C). AT2 signals were only observed after reverse transcription of the RNA indicating that the signals were not due to DNA contamination. As expected, untransfected cells did not exhibit AT2 signals, but were positive in the β actin control. This confirmed the specificity of the AT2 primer pair for the introduced human AT2 transgene.

The G allele of the AT2 1675 G/A PM expresses higher protein levels than the A allele: In contrast to the identical mRNA splice pattern, luciferase activities driven by the two AT2 minigene/luciferase constructs were significantly different (Fig. 4). In rat vascular smooth muscle cells luciferase activities expressed from the G allele construct were 54.7%±17.0 (p<0.005, n=10) higher, in PC12W cells 37.7%±9.9 higher (p<0.005, n=8) and in HT1080 cells 25.8%±6.6 (p<0.005, n=10) higher than those expressed by the A allele construct (Fig. 4). Since in this cell culture model luciferase levels may be regarded as representatives of
AT2 protein levels *in vivo*, the data imply that the 1675 G/A PM may modulate AT2 protein expression.
Discussion

Two independent studies associated the human AT2 gene 1675 G/A PM with left ventricular structure and recurrent coronary ischemia (30;31). Since the PM is located in an intronic splice branch site, it was postulated that it might affect pre-mRNA splicing and thereby AT2 receptor expression. However, this hypothesis had not been verified convincingly. Therefore, we determined the effect of the PM systematically 1. in *in vitro* transfection experiments, and 2. *ex vivo* by comparison of the myocardial AT2 splice patterns with the genotypes of 12 patients with heart failure.

In spite of careful investigation by many researchers, the function of AT2 in cardiovascular cells has not yet been completely clarified. In earlier studies we overexpressed the human AT2 receptor in porcine cardiac fibroblasts (PC12) and human umbilical vein endothelial cells (HUVEC). Overexpression of AT2 receptors in these cells, which did not exhibit a native AT2, led to a six- to ten-fold higher expression of AT2 in comparison with endogenous AT1 receptors. Stimulation of the overexpressed AT2 receptor in fibroblasts inhibited tyrosine phosphatase activity but had no significant effect on fibroblast functions such as collagen synthesis, migration or proliferation. However, inhibition of phosphotyrosine phosphatase Ib which regulates insulin signaling was achieved by AT2 stimulation (41).

At first, however, we performed data base search to exactly localize the PM: it is positioned in the 152-bp intron 1 of the AT2 gene, 30 bp upstream of the splice acceptor site, in a sequence motif that is indeed homologous to the splice branch site consensus (Fig. 1) (42;43). This consensus sequence is poorly defined: The only nucleotide that is strictly conserved is the A nucleotide at which the lariat-splicing intermediate is formed (44). In contrast, the PM is 5´adjacent to this adenosine at a position where a purin nucleotide (A or G) is conserved.
Therefore, it was questionable whether the PM might affect the AT2 mRNA splice pattern. We suggested that in case of a complete inactivation of the splice branch site by one of the alleles skipping of the small exon 2 and a splice pattern shift towards a preponderance of the small splice variant exon 1/3 might occur. As we demonstrated previously, this would result in an increase of AT2 receptor protein expression (36).

In earlier studies we determined the genotype distribution and allele frequency of the AT2 gene in controls and patients with heart disease. No sex difference in gene expression was found (45). However, in myocardial tissue samples (atria and ventricles) of 12 explanted hearts from genotyped patients (7 A, 5 G allele carriers), in all three cell types transfected with the human AT2 constructs as well as in myometrial samples, which were used as a source of AT2 mRNA in our previous work (data not shown), we identified the same pattern of AT2 mRNA expression: the mRNA consisting of exon 1/2/3 was the most abundant variant, whereas the transcript exon 1/3 was rare and sometimes below the detection limit, when total AT2 mRNA expression was low.
These data are in contrast to a study by Nishimura and colleagues (35), who identified in primary fibroblasts derived from male and therefore hemizygous individuals and in uterine tissue samples from homozygous women AT2 genotype-dependent differences in the AT2 splice pattern. In this study the A allele carriers expressed the splice variant exon 1/2/3 only and at a high expression level, whereas the G allele carriers expressed splice variant exon 1/3 only and at a low level. The authors concluded - but did not confirm by protein expression data - that the G allele may be associated with a reduced AT2 receptor expression. They did not explain, why a splice pattern shift should result in such a pronounced difference in total AT2 mRNA expression.

In contrast, in the present study luciferase expression levels from the AT2 minigene constructs implied that the G allele carriers may exhibit a moderately higher receptor protein expression than the A allele carriers.

Nishimura and colleagues found a strong association of the G allele with two forms (ureteropelvic junction stenosis or atresia and multicystic dysplastic kidneys) of congenital anomalies of the kidney and urinary tract (CAKUT) within two cohorts of male American caucasians (13 patients / 31 controls) and male German caucasians (23 patients / 24 controls) (35). However, this association was not confirmed in a cohort of 66 japanese boys with CAKUT (46). Modifier genes were suggested to contribute to the incomplete penetrance of this rare disease and may explain why the association was not identified in other study populations.

In one of the studies demonstrating an association of the AT2 PM with left ventricular wall thickness, this effect was only detectable in young male individuals with mild hypertension (30). This may suggest that AT2 effects only become relevant in pathological situations, presumably because the receptor is upregulated in the remodeling cardiovascular system (27-
29), or on a certain, not yet defined, genetic background which includes the possibility that
the PM is in linkage disequilibrium with another not yet identified PM. The latter may be
supported by the observation that some inbred AT2 knock-out mouse strains tend to develop
CAKUT (35), but most do not (18;19).
In conclusion, our data demonstrate that the AT2 1675 PM is not associated with a qualitative
modification of AT2 pre-mRNA splicing, i.e. a splice pattern shift, but with different AT2
protein expression levels. Although the precise mechanism underlying this difference remains
to be determined, we found that it was not related to the promoter-like activity of intron 1
which was identified in a previous work (35) (data not shown). Rather it is conceivable that a
slightly higher affinity of the splicing factors to the G allele may result in a more efficient pre-
mRNA processing and thereby protein expression (47). The moderately higher AT2
expression which may occur in the myocardium and the vasculature of G allele carriers,
particularly under pathological conditions associated with an increased AT2 expression, may
exert protective effects and reduce cardiac hypertrophy and coronary artery remodeling.

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Prof. S. Bottari, INSERM, Grenoble, France for providing PC12W cells.
References


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### B)

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Figure 1
Figure 2

A)

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300 bp- 200 bp- 100 bp-

Ratio of splice variants
92% Ex1/2/3
8% Ex1/3

Genotype

B)

Figure 2
A) AT2 minigene/luciferase construct

+ Primer - Primer

Plasmid control 1/2/3

Plasmid control 1/3

B)

RT+ RT- plasm co

M A G co A G co 1/3 1/2/3

300 bp-200 bp-100 bp-

hAT2 32 c.

300 bp-200 bp-

β-Actin 26 c.

C)

RT+ RT- plasm co

M A G co A G co 1/3 1/2/3

300 bp-200 bp-100 bp-

hAT2 30 c.

300 bp-200 bp-

β-Actin 26 c.
Figure 3

Luciferase activity (RLU)

rVSMC
n=10
p<0.01

PC12W
n=8
p<0.01

HT1080
n=10
p<0.01

Figure 4
Figure legends

Fig. 1. Structure of the human AT2 gene and localization of the polymorphism.

A, schematic drawing according to Martin and Elton (39) of the human AT2 gene with its two introns and three exons and its mRNA splice variants (not to scale). B, localization of the 1675 G/A PM (highlighted) within the small intron 1 of the AT2 gene. Open boxes mark the flanking exons 1 and 2. The shaded box marks a sequence motif with enhancer activity which was identified in a previous study (40). Below: comparison between the AT2 sequence surrounding the PM and the splice branch site consensus. Note that the PM localizes to the nucleotide position 5´ adjacent to the highly conserved A residue at which the lariat-splicing intermediate is formed.

Fig. 2 Myocardial AT2 mRNA splice pattern of explanted human hearts and corresponding AT2 genotypes.

A, example of a RT PCR analysis demonstrating the AT2 splice variants exon 1/2/3 (upper band) and exon 1/3 (lower band) in myocardial tissue samples from explanted human hearts (reproduced with permission from (36). For the present study the genotypes of the patients were determined (for summary see table 1). Note that the splice pattern is identical for both genotypes. In samples with lower total AT2 mRNA expression the exon 1/3 variant approaches the detection limit. HF: heart failure, co control, LV left ventricle, RV right ventricle, RA right atrium, PDH co pyruvic dehydrogenase control, M 100 bp DNA size marker. B, example of a single strand conformational polymorphism (SSCP) PCR analysis for
the determination of the AT2 1675 PM. Arrows indicate the bands which allow differentiation of the two alleles.

**Fig. 3 AT2 mRNA splice variants in cells transfected with AT2/minigene luciferase constructs expressing either AT2 allele.**

A, schematic drawing of the AT2 minigene/luciferase reporter constructs used for transfection experiments. Since the constructs contain the complete transcribed, but not translated, AT2 sequence up to the translation start codon in exon 3, mRNA splicing and the postulated regulation of splicing by the PM should be identical to the endogenous AT2 alleles; B and C, results of RT PCRs demonstrating identical AT2 mRNA splice patterns in B, HT1080 cells and C, PC12W cells that were transiently transfected with the minigene constructs, and control PCRs for β actin. RT+ reverse transcription prior to PCR, RT- no reverse transcription prior to PCR, M 100 bp DNA size marker, the letters A and G indicate the allele of the AT2 minigene construct used for transfection, co untransfected cells, H2O control without cDNA, plasm co control PCR amplification of plasmids which contain the already spliced cDNA sequences (1/3 = exon 1/3, 1/2/3 = exon 1/2/3), β actin PCR served as control for RNA input and quality. 32 c., 26 c. and 30 c. indicates the number of PCR cycles. Note that the AT2 primer pair used in this assay is different from that used in Fig. 2 A resulting in different PCR product sizes.

**Fig. 4 Luciferase activities expressed from minigene constructs carrying either allele of the PM.**
Luciferase activities (relative light units = RLU) expressed from the two constructs in transfected rat vascular smooth muscle cells (rVSMC), rat pheochromocytoma PC12W cells, and human fibrosarcoma HT1080 cells. Luciferase activities reflect total AT2 protein expression levels driven by the respective AT2 allele. The activity of the A allele construct was set to 100%, activities of the G allele construct are given as mean relative light units (RLU)±SEM, n = number of independent transfection experiments, **p<0.005.
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Table 1 AT2 genotype of 12 patients with known myocardial AT2 splice pattern. Twelve of the 14 patients with end-stage heart failure who had undergone orthotopic heart transplantation and whose explanted hearts were analyzed for AT2 mRNA splice pattern in a previous study (31) were now genotyped. Comparison of the RT-PCR results (Fig. 2 A) with the respective genotype revealed that the myocardial splice pattern was not affected by the genotype. (Abbreviations: DCM-dilated cardiomyopathy, CHD-coronary heart disease, MI-myocardial infarction, AS-aortic stenosis, AI-aortic insufficiency)