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Wackenfors, Angelica; Pantev, Emil; Emilson, Malin; Edvinsson, Lars; Malmsjö, Malin

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PO Box 117
221 00 Lund
+46 46-222 00 00

Angiotensin II Receptor mRNA Expression and Vasoconstriction in Human Coronary Arteries: Effects of Heart Failure and Age

Angelica Wackenfors, Emil Pantev, Malin Emilson, Lars Edvinsson and Malin Malmström

Division of Experimental Vascular Research, Department of Internal Medicine, Lund University Hospital, Lund, Sweden

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Abstract: Angiotensin II is a potent vasoconstrictor that is implicated in the pathogenesis of hypertension, heart failure and atherosclerosis. In the present study, angiotensin II receptor mRNA expression levels were quantified by real-time polymerase chain reaction and the vasocontractile responses to angiotensin II were characterised by *in vitro* pharmacology in endothelium-denuded human coronary arteries. Angiotensin II type 1 (AT₁) and type 2 (AT₂) receptor mRNA expression levels were significantly down-regulated in arteries from patients with heart failure as compared to controls. The angiotensin II-induced vasoconstriction diminished with increasing age in patients with heart failure ($r^2=0.31$, $P<0.05$). Also, the AT₁ receptor mRNA expression levels decreased with increasing age in patients with heart failure ($r^2=0.74$, $P<0.05$), while no such correlation could be shown in the control group ($r^2=0.04$, $P=n.s.$). The AT₂ receptor mRNA expression levels did not correlate with age in patients with heart failure or controls. In conclusion, the diminished angiotensin II vasoconstriction with age in heart failure patients is most likely due to a lower density of AT₁ receptors and may result from a longer period of exposure to heart failure in older patients.

Angiotensin II is the principal effector molecule of the renin-angiotensin system. Angiotensin II is produced by the action of renin on angiotensinogen to form angiotensin I and its subsequent conversion to the biologically active peptide by angiotensin-converting enzyme. The formation of angiotensin II has been demonstrated in the systemic circulation and in a number of local tissues including the human vasculature (Kifor & Dzau 1987). Angiotensin II is a potent vasoconstrictor that regulates regional blood flow and a growth factor that elicits vascular smooth muscle cell proliferation (Daemen *et al.* 1991; Simon & Altman 1992; Weber 2000).

In the human vasculature, the angiotensin II effects are mediated by the angiotensin II type 1 (AT₁) and type 2 (AT₂) receptors. These are G-protein coupled receptors that have been identified, cloned and sequenced in man (Furuta *et al.* 1992; Tsuzuki *et al.* 1994). The AT₁ and AT₂ receptors are distributed heterogeneously in human tissues including blood vessels and the heart (Kim & Iwao 2000). Angiotensin II-induced vasoconstriction in human arteries is mediated primarily by AT₁ receptors on smooth muscle cells (Garcha *et al.* 1999; Ytterberg & Edvinsson 2001). The AT₂ receptors are mainly believed to induce endothelium-dependent dilatation by release of nitric oxide and prostaglandins (Carey *et al.* 2000). In human coronary arteries, AT₂ receptors on smooth muscle cells mediate vasoconstriction (Pantev *et al.* 2002).

The angiotensin II receptor expression changes during the development of cardiovascular pathology such as vascular injury and cardiac remodelling (Masaki *et al.* 1998; Matsubara 1998; Akishita *et al.* 2000; Katugampola & Davenport 2000). Even though inhibitors of the renin-angiotensin system are increasingly used for the treatment of heart failure and hypertension, data on angiotensin II receptor mRNA expression and function in the human vasculature is scarce. The hypothesis of the present study is that angiotensin II receptor expression and function in human coronary arteries varies between patients and is dependent on the disease state. The aim of the study was to evaluate which background factors that are of importance for the variation of the angiotensin II receptor expression and function. AT₁ and AT₂ receptor mRNA expression levels were quantified by real-time polymerase chain reaction in endothelium-denuded human coronary arteries from patients with heart failure as compared to controls. Furthermore, the influence of age on the angiotensin II-mediated vasoconstriction was evaluated.

Materials and Methods

Limitations of the study. The supply of human material is limited. Whole coronary arteries can be received only from explanted hearts during heart transplantation. *In vitro* pharmacology experiments are therefore run on arteries from heart failure patients (New York Heart Association (NYHA) II–IV), and no proper controls for this group can be obtained. For obvious reasons, collection of human coronary arteries from explanted hearts takes time, thereby the limited size of material. For ethical reasons, only biopsies of coronary arteries can be obtained during autopsy and the material is

Author for correspondence: Angelica Wackenfors, Experimental Vascular Research, BMC A13, SE-221 84 Lund, Sweden (fax +46 46 222 0616, e-mail angelica.wackenfors@med.lu.se).

Table 1.

Baseline characteristics	
Mean age, range	60 years, 21–86 years
Gender (Men/women)	62%/38%
Smokers	50%
<i>Clinical diagnosis</i>	
Dilated cardiomyopathy	32%
Restrictive cardiomyopathy	24%
Ischemic cardiomyopathy	32%
Diabetes mellitus	12%
Hypertension	8%
Hyperlipidemia	12%
Tricuspid insufficiency	4%
<i>Heart failure</i>	
NYHA II	24%
NYHA III	52%
NYHA VI	24%
Mean EF, range	22%, 8–55%

Baseline characteristics for the patients with heart failure. NYHA=New York Heart Association; EF=ejection fraction.

only sufficient for real-time polymerase chain reaction, and not *in vitro* pharmacology experiments.

Tissue collection. Biopsies from coronary arteries, used for real-time polymerase chain reaction experiments, were obtained *post mortem* during autopsy. Included in the study were eight patients that died from heart failure (age 36–80). The patients had suffered from heart failure for a longer period of time. Nine control patients were included. These did not have any known cardiovascular disease at the time of death (controls, age 40–83). In the control group the cause of death was pneumonia (three patients), stroke (three patients), acute respiratory distress syndrome (one patient), sepsis (one patient) and trauma (one patient). The control patients did not have heart failure diagnosed by the time of death, and no atherosclerotic lesions could be identified in the coronary arteries when dissected under a microscope. The relation between the time of death and the time of biopsy was 8–56 hr, and did not differ between the heart failure and the control group. After removal of the endothelium, the vessels were snap frozen in liquid nitrogen and stored at -80° .

For the *in vitro* pharmacology experiments, a section of the left anterior descending artery was obtained from 17 human hearts explanted in the process of heart transplantation (age 21–62). None of the patients had been treated with AT₁ receptor blockers before surgery. The vessels were removed from the patients and immersed into cold bicarbonate buffer solution (for composition, see below), transported to the laboratory on ice and immediately used for further experiments. For baselines characteristics for the patients with heart failure (table 1).

***In vitro* pharmacology.** The arteries were dissected free from adhering tissue and the luminal side was gently rubbed with a metal wire to disrupt the endothelium. The arteries were then cut into cylindrical segments (3–4 mm long). The segments were mounted on two L-shaped metal prongs, one of which was connected to a force displacement transducer for continuous recording of the isometric tension (Högstätt *et al.* 1983). The mounted artery segments were immersed in temperature controlled (37°) tissue baths containing a bicarbonate based buffer solution (for composition, see below), which was continuously gassed with 5% CO₂ in O₂ resulting in a pH of 7.4. Eight to sixteen segments were studied at the same time in separate tissue baths. The segments were allowed to stabilise at a resting tension of 4 mN for 1 hr before the experiments were started. The contractile capacity of each vessel segment was examined by exposure to a K⁺ rich (63.5 mM) buffer solution. Cumulative concentration-response curves were constructed for all experi-

ments by the addition of increasing concentrations of angiotensin II (0.01 nM–5 μ M). The experiments were terminated by the addition of the endothelium-dependent vasodilator adenosine 5'-O-thiodiphosphate (10 μ M) to check that the endothelium was properly removed (Malmsjö *et al.* 2000). Abolished dilatation indicated a properly removed endothelium.

Real-time polymerase chain reaction. Real-time polymerase chain reaction was run on the endothelium-denuded human coronary arteries. Reverse transcription of total RNA to cDNA was carried out using the GeneAmp RNA polymerase chain reaction kit in a Perkin-Elmer DNA Thermal cycler (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). First strand cDNA was synthesized from 0.5–1 μ g total RNA in a 100 μ l reaction using random hexamers as primers. Real-time polymerase chain reaction was performed in a GeneAmp 5700 Sequence Detection System using the GeneAmp SYBR[®] Green kit (Perkin-Elmer, Applied Biosystems Foster City, CA, USA) with the cDNA synthesized above as template in a 50 μ l reaction. A no-template control was included in all experiments. The GeneAmp 5700 Sequence Detection System monitors the am-

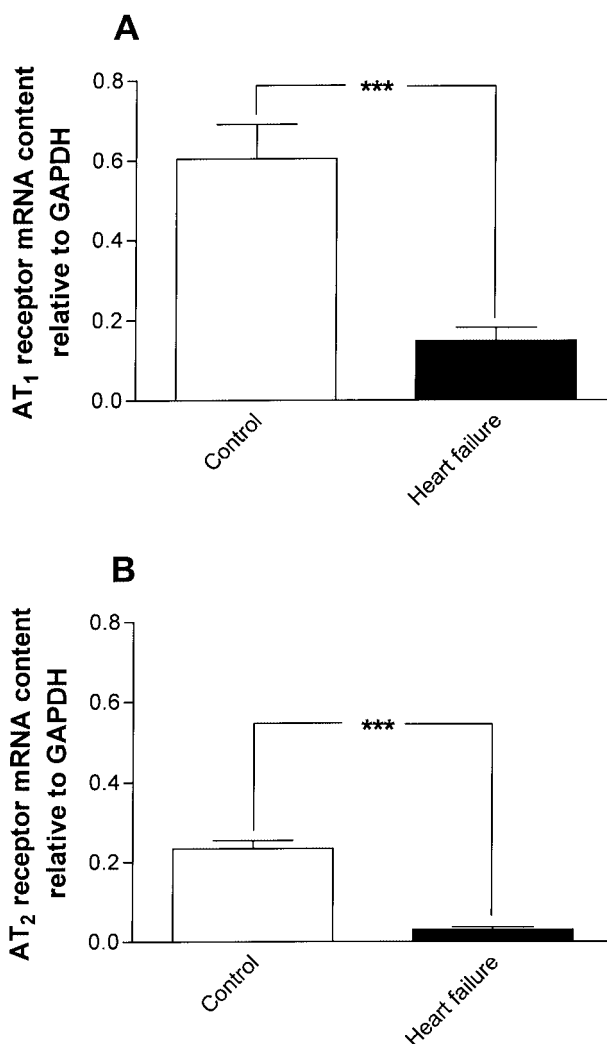


Fig. 1. AT₁ (A) and AT₂ (B) receptor mRNA expression levels assessed by real-time polymerase chain reaction in human coronary arteries from patients with heart failure and controls. Values are presented as mean values \pm S.E.M relative to the GAPDH levels. Statistical significance was accepted when $P < 0.05$, using Student's *t*-test.

plification of DNA in real-time using an optic imaging system, via the binding of a fluorescent dye to double-stranded DNA. Specific primers for the human AT₁ and AT₂ receptors were designed as follows:

AT₁ receptor

forward; 5'-ACC TGG CTA TTG TTC ACC CAA-3'

reverse; 5'-ACA AGC ATT GTG CGT CGA AG-3'

AT₂ receptor

forward; 5'-CCT CGC TGT GGC TGA TTT ACT C-3'

reverse; 5'-CTT TGC ACA TCA CAG GTC CAA-3'

β -Actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as references, since they are continuously expressed in constant amounts in cells.

The real-time polymerase chain reaction was performed with the following profile: 50° for 2 min., 95° for 10 min., 40 cycles of 95° for 15 sec. and 60° for 1 min. To prove that the cDNA of β -actin, GAPDH, AT₁ and AT₂ receptors were amplified with the same efficiency during the real-time polymerase chain reaction experiments, standard curves for each primer pair were made (fig. 1) in which the C_T values were plotted against cDNA concentration on the basis of the following equation: $C_T = [\log(1+E)]^{-1} \log(\text{concentration})$, where C_T is the number of polymerase chain reaction cycles performed in 1 sample at a specific point of time, and E is

the amplification efficiency with an optimal value of 1. To prove that each primer pair generates only one polymerase chain reaction product, an agarose gel electrophoresis with the polymerase chain reaction products was run. The lengths of the products were 101 base pairs for both AT₁ and AT₂. This corresponds to the expected size of the AT₁ and the AT₂ cDNA from the gene bank was examined with the program Primer Express 2.0.

Drugs and solutions. The bicarbonate buffer solution for the *in vitro* pharmacology experiments was of the following composition in mM: 119 NaCl, 15 NaHCO₃, 4.6 KCL, 1.2 MgCl₂, 1.2 NaH₂PO₄, 1.5 CaCl₂ and 5.5 glucose. Adenosine 5'-O-thiodiphosphate (Sigma Co., USA) was dissolved in 0.9% saline. Angiotensin II (Sigma Co., USA) was dissolved in 0.1% bovine serum albumin. Oligonucleotides and reagents for the real-time polymerase chain reaction assay were purchased from Perkin-Elmer, Applied Biosystems, Foster City, CA, USA.

Calculations and statistics. *In vitro* pharmacology: All calculations and statistics were performed using GraphPad 4.0 software. E_{max} refers to the maximum contraction calculated as percent of the contractile capacity of 63.5 mM K⁺. The negative logarithm of the drug concentration that elicited 50% contraction (pEC₅₀) was determined by linear regression analysis using the values immediately above and below half-maximum response. Statistical significance

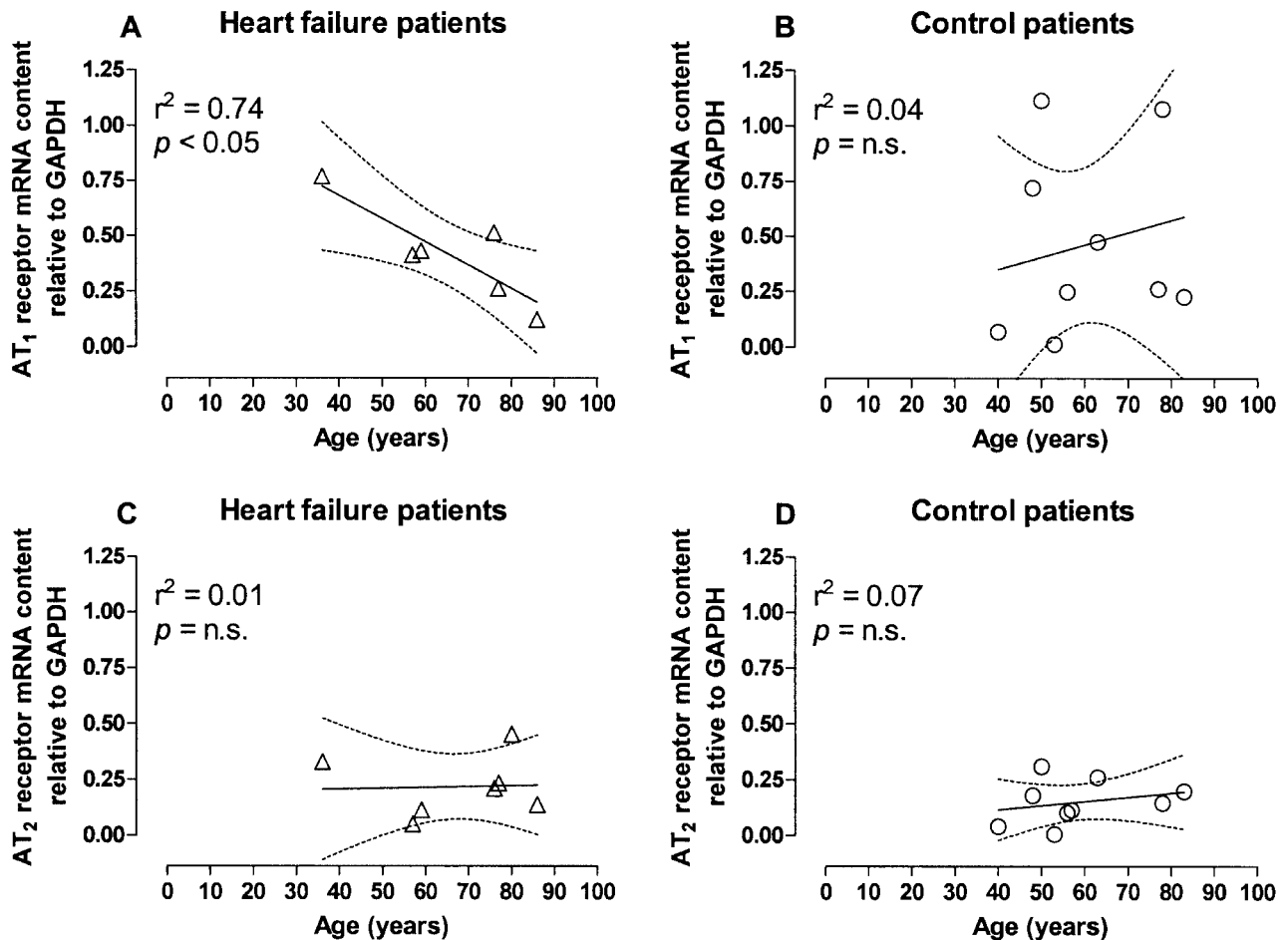


Fig. 2. AT₁ (A and B) and AT₂ (C and D) receptor mRNA expression levels assessed by real-time polymerase chain reaction in human coronary arteries from patients with heart failure (A and C) and controls (B and D). The results were correlated to the patients' ages and illustrated with a linear regression line and a 95% confidence interval. Values are presented as mean values \pm S.E.M relative to the GAPDH levels. Statistical significance was accepted when $P < 0.05$, using Student's t-test.

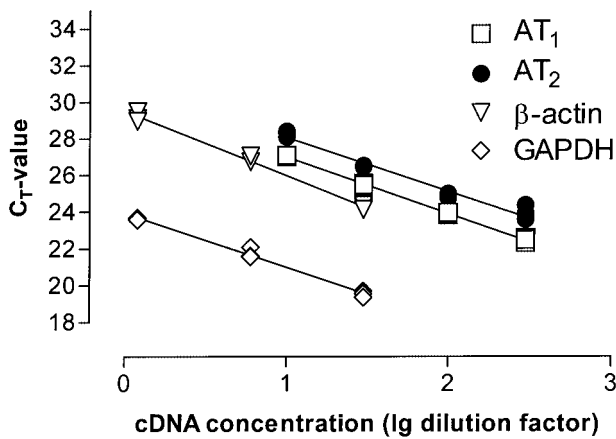


Fig. 3. Standard curves for the AT₁ receptor, the AT₂ receptor and the two housekeeping genes β -actin and GAPDH.

was accepted when $P < 0.05$, using Student's t-test. Values are presented as means \pm S.E.M.

Real-time polymerase chain reaction: 22 experiments were performed on eight arteries from patients with heart failure, 22 experiments were performed on nine arteries from patients without known cardiovascular disease. The amount of AT₁ and AT₂ receptor mRNA was calculated as relative to the amount of GAPDH or β -actin mRNA in the same sample by the formula: $X_0/R_0 = 2^{CtR - CtX}$, where X_0 = original amount of angiotensin II receptor mRNA, R_0 = original amount of GAPDH or β -actin mRNA, CtR = C_T -value for GAPDH or β -actin and CtX = C_T -value for the angiotensin II receptor. Statistical significance was accepted when $P < 0.05$, using Student's t-test. Values are presented as means \pm S.E.M relative to GAPDH mRNA levels.

Regression analyses and Pearson correlation analyses were performed to determine how well two variables vary together. r^2 is the coefficient of determination and is the fraction of variance in the two variables that is shared.

Ethics. The project was approved by the Ethics Committee of Lund University in Sweden and Szeged University in Hungary, conforms to the principles outlined in the Declaration of Helsinki.

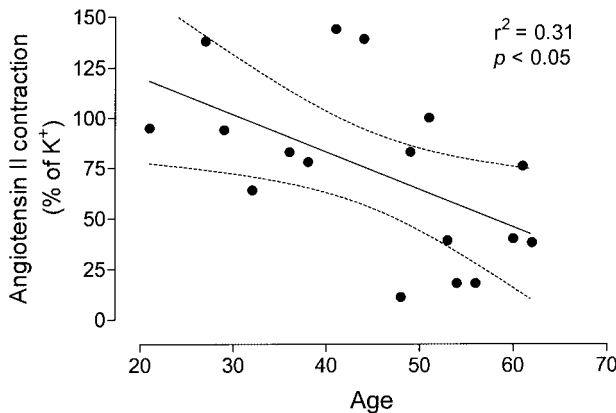


Fig. 4. The maximum vasocontractile response induced by angiotensin II in human coronary arteries. Correlation with patient age, illustrated with a linear regression line and a 95% confidence interval. Statistical significance was accepted when $P < 0.05$, using Student's t-test.

Results

Real-time polymerase chain reaction.

The AT₁ and AT₂ receptor mRNA expression levels were lower in coronary arteries from patients with heart failure as compared to controls ($P < 0.001$, fig. 1A and B). There was no difference in age between the heart failure patients and the controls (heart failure; 63 ± 8 years, controls; 59 ± 3 years, $P = n.s.$). The AT₁ receptor mRNA expression levels decreased with increasing age in patients with heart failure ($r^2 = 0.74$, $P < 0.05$), while no correlation could be shown in the control group ($r^2 = 0.04$, $P = n.s.$, fig. 2A and B). The AT₂ receptor mRNA expression levels did not correlate with increasing age in patients with heart failure ($r^2 = 0.01$, $P = n.s.$) or controls ($r^2 = 0.07$, $P = n.s.$, fig. 2C and D).

mRNA expression levels were higher for the AT₁ than for the AT₂ receptor, both in arteries from patients with heart failure and controls (fig. 1A and B). The standard curves for the AT₁, AT₂, β -actin and GAPDH cDNA primer pairs are shown in fig. 3. Similar patterns of AT₁ and AT₂ receptor mRNA expression could be shown when using β -actin for reference gene as compared to GAPDH (data not shown), indicating that these genes were trustworthy as references. Electrophoresis of the polymerase chain reaction products demonstrated that each primer pair generated only the expected product with a length of 101 base pairs for AT₁ and 101 base pairs for AT₂. The no-template control showed no signs of contaminating nucleic acids (data not shown).

In vitro pharmacology. Coronary arteries for the *in vitro* pharmacology experiments were obtained during heart surgery from patients with heart failure (NYHA II–IV). In these arteries, the efficacy of the contractile response to angiotensin II declined with increasing age ($r^2 = 0.31$, $P < 0.05$, fig. 4 and 5), while the potency was unchanged ($pEC_{50} = 8.1 \pm 0.2$, $r^2 = 0$, $P = n.s.$), indicating receptor down-regulation. The patients' ages ranged from 21 to 62 years.

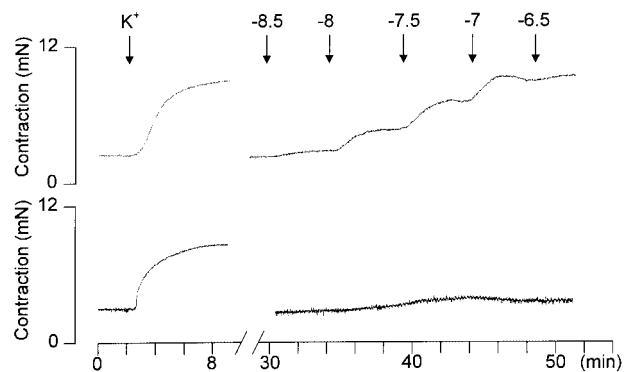


Fig. 5. Representative examples of contractions induced by a single dose of K^+ (63.5 mM) and then increasing concentrations of angiotensin II ($-\log M$), in coronary arteries from patients of different age. **A:** 21 years of age. E_{max} for angiotensin II = 95% of K^+ . **B:** 56 years of age. E_{max} for angiotensin II = 18% of K^+ .

The K^+ -induced contraction was not affected by age ($P = n.s.$), indicating intact smooth muscle cell function.

Discussion

The levels of AT_1 and AT_2 receptor mRNA were reduced in coronary artery smooth muscle cells from patients with heart failure as compared to controls. This may result in a less efficacious angiotensin II response. Unfortunately, functional studies can not be performed on arteries obtained during *post mortem* autopsy due to limited tissue supply. Many studies have been performed in animals to evaluate the effect of angiotensin II in the vasculature during heart failure. Increased as well as unaltered or impaired responses to angiotensin II have been described, and the results do not provide a consistent picture (Didion *et al.* 1997; Stassen *et al.* 1997; Ikenaga *et al.* 1999; Miller *et al.* 2000; Touyz & Schiffrin 2000; Gschwend *et al.* 2003). In this respect, the vascular adaptation mechanisms may differ depending on duration and severity of heart failure and the arterial bed investigated.

The down-regulation of angiotensin II receptor mRNA expression levels in heart failure is supported by previous data showing that when coronary arteries are obtained mainly from humans dead in accidents, the pEC_{50} value for angiotensin II is 9.3 (Holmgren *et al.* 1998). When coronary arteries from explanted failing hearts are studied the pEC_{50} value is 7.7 (Pantev *et al.* 2002) and 8.1 (in the present study). In the present study, 32% of the patients with heart failure had the diagnosis ischaemic heart disease. It has been shown before by autoradiography that AT_1 receptors are down-regulated in coronary arteries from humans with ischaemic heart disease (Katugampola & Davenport 2000). Down-regulation of angiotensin II receptors might make the coronary arteries less prone to develop spasm and atherosclerotic plaques and thus restore vital blood circulation to the failing heart despite the elevated circulating levels of angiotensin II (Zimmerman & Davisson 2004).

The cause of angiotensin II receptor changes in heart failure is not known although alterations in the neurohormonal pathways involving both the renin-angiotensin system and sympathetic nervous system may be of importance. Constricting factors (e.g. thromboxane A₂, angiotensin II and endothelin-1), vasodilating factors (e.g. nitric oxide) and inflammatory hormones (cytokines) are released locally by the endothelium, nerve terminals, platelets and leukocytes during heart failure. These have been suggested to participate in vascular remodelling and feedback to the forebrain and hypothalamic centres to stimulate further sympathetic activation (Fang & Marwick 2002; Felder *et al.* 2003). Increased systemic or local production of angiotensin II may have stimulated receptor down-regulation via a negative feed-back system. It has been shown before that exposure to angiotensin II induces down-regulation of AT_1 receptor mRNA in rat aortic vascular smooth muscle cells and cultured rat glomerular cells (Makita *et al.* 1992; Lassegue *et al.* 1995).

In vitro pharmacological studies were performed on coronary arteries from hearts explanted during the procedure of heart transplantation (NYHA II–IV). The present results show that the angiotensin II-induced vasoconstriction declines with increasing age in human coronary arteries from patients with heart failure (table 1). The efficacy of the contraction was decreased while the potency was unaltered, which may reflect a change in receptor expression or intracellular pathways according to receptor theories (Black & Leff 1983). Previous studies on isolated arteries have demonstrated a reduced angiotensin II contraction in old animals (Marin 1995). The K^+ -induced vasoconstriction was not altered by age in the present study, indicating that the contractile machinery is unaffected, which has been shown by others (Marin 1995).

The mechanisms responsible for the decreased angiotensin II-induced vasoconstriction in old humans are not known. Other contractile receptors, e.g. adrenoceptors, have been shown to be down-regulated in arteries from old humans (Marin 1995). It is believed that the adrenoceptors are desensitised due to elevated levels of catecholamines in plasma (Marin 1995). Aging induces over-expression of tissue angiotensin-converting enzyme (Belmin *et al.* 1994). Most of the angiotensin II that induces vasoconstriction is synthesised locally in the vascular wall by tissue renin-angiotensin system (Touyz & Schiffrin 2000). Presumably this results in increased angiotensin II concentrations in the vascular wall and subsequent angiotensin II receptor down-regulation.

In previous *in vitro* pharmacology studies, it has been shown that the angiotensin II-induced vasoconstriction in human coronary arteries is mediated by AT_1 receptors, since the response was inhibited by candesartan, and AT_2 receptors, since the response was inhibited by and PD123319 (Pantev *et al.* 2002). When analysing the present real-time polymerase chain reaction experiments, both AT_1 and AT_2 receptor mRNA were detected. The AT_1 receptor mRNA expression levels decreased with increasing age in patients with heart failure, while no such correlation could be shown for the AT_2 receptor. Thus, the diminished angiotensin II vasoconstriction with increasing age in heart failure patients is most likely due to a lower density of AT_1 receptors. The AT_1 receptor mRNA expression levels were decreased in old patients in the heart failure group, but not in the control group. These results suggest that the decrease AT_1 receptor mRNA expression and angiotensin II-induced vasoconstriction with increasing age may be a result of a longer period of exposure to heart failure in older patients, although other factors that change with increasing age can not be excluded.

In conclusion, the present study demonstrates for the first time that AT_1 and AT_2 receptor mRNA expression levels are decreased in arteries from patients with heart failure as compared to controls. AT_1 receptor mRNA was expressed in higher amounts than AT_2 receptor mRNA, which is in accordance with the assumption that AT_1 receptors are of major importance in mediating the vasocontractile effects

of angiotensin II (Pantev *et al.* 2002). AT₁ and AT₂ receptor mRNA expression levels were significantly down-regulated in arteries from patients with heart failure as compared to controls. The angiotensin II-induced vasoconstriction diminished with increasing age in patients with heart failure. Also, the AT₁ receptor mRNA expression levels decreased with increasing age in patients with heart failure, while no such correlation could be shown in the control group. Thereby, the diminished angiotensin II vasoconstriction with increasing age in heart failure patients is most likely due to a lower density of AT₁ receptors and may result from a longer period of exposure to heart failure in older patients. Since the angiotensin II-induced vasoconstriction decreases with increasing age, a treatment with angiotensin-converting enzyme or an AT₁ receptor inhibitor may be less effective in elderly patients. Tailoring the treatment, e.g. treating younger patients with heart failure or hypertension with angiotensin-converting enzyme or AT₁ receptor inhibitor while using different pharmaceutical agents for elderly patients, may be more beneficial.

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