Clinical Research

Cardiac Angiotensin II Formation in the Clinical Course of Heart Failure and Its Relationship With Left Ventricular Function

Gian Gastone Neri Serneri, Maria Boddi, Ilaria Cecioni, Simone Vanni, Mirella Coppo,
Maria Letizia Papa, Brunella Bandinelli, Iacopo Bertolozzi, Gianluca Polidori, Thomas Toscano,
Massimo Maccherini, Pietro Amedeo Modesti

Abstract—In 76 patients with heart failure (HF) (New York Heart Association [NYHA] classes I through IV) and in 15 control subjects, cardiac angiotensin II (Ang II) generation and its relationship with left ventricular function were investigated by measuring aorta–coronary sinus concentration gradients of endogenous angiotensins and in a part of patients by studying ^{125}I-labeled Ang I kinetics. Gene expression and cellular localization of the cardiac renin-angiotensin system components, the density of AT_1 and AT_2 on membranes and isolated myocytes, and the capacity of isolated myocytes for synthesizing the hypertrophying growth factors insulin-like growth factor-I (IGF-I) and endothelin (ET)-1 were also investigated on 22 HF explanted hearts (NYHA classes III and IV) and 7 nonfailing (NF) donor hearts. Ang II generation increased with progression of HF, and end-systolic wall stress was the only independent predictor of Ang II formation. Angiotensinogen and angiotensin-converting enzyme mRNA levels were elevated in HF hearts, whereas chymase levels were not, and mRNAs were almost exclusively expressed on nonmyocyte cells. Ang II was immunohistochemically detectable both on myocytes and interstitial cells. Binding studies showed that AT_1 density on failing myocytes did not differ from that of NF myocytes, with preserved AT_1/AT_2 ratio. Conversely, AT_1 density was lower in failing membranes than in NF ones. Ang II induced IGF-I and ET-1 synthesis by isolated NF myocytes, whereas failing myocytes were unable to respond to Ang II stimulation. This study demonstrates that (1) the clinical course of HF is associated with progressive increase in cardiac Ang II formation, (2) AT_1 density does not change on failing myocytes, and (3) failing myocytes are unable to synthesize IGF-I and ET-1 in response to Ang II stimulation. (***Circ Res***. 2001;88:961-968.)

Key Words: angiotensins ■ congestive heart failure ■ AT receptors ■ failing myocytes ■ cardiomyopathy

Conclusive evidence has been presented that all of the components required for angiotensin II (Ang II) production are present in the human heart[1] and that Ang II is continuously formed by cardiac tissues in healthy subjects.[2] However, few studies have investigated the cardiac renin-angiotensin system (RAS) in human hypertrophy and heart failure (HF), as opposed to the large number of experimental studies performed in cell culture systems and experimental models (for studies, see Wollert and Drexler[3]). In human explanted failing hearts, levels of angiotensin-converting enzyme (ACE) mRNA,[4] ACE activity, and ACE binding sites[5] have been found to be increased as compared with control hearts. Levels of prorenin, renin, and ACE measured with enzyme kinetics are higher in failing than in control hearts.[6] Although these studies provide important results, demonstrating that cardiac RAS is activated in explanted failing hearts, they do not provide information either about

cardiac Ang II production during the clinical course of HF or about the relationship between Ang II and cardiac function. Moreover, these studies do not answer the critical question as to whether the enhanced cardiac expression of ACE actually promotes an increased production of Ang II, because several groups have challenged the notion that ACE is the major Ang II–forming enzyme in the human heart.[7,8] A second open question is the identification of the cell types that actually express Ang II receptors in the failing myocardium and constitute the potential targets for Ang II receptor–mediated effects. Several groups have investigated the density of Ang II receptor subtypes in human failing hearts and have found a reduction in AT_1,[5,9–11] either without changes in AT_2[9,10] or with an increase in them.[5,11] However, these studies were only performed on myocardial homogenates, and no information is available on the regulation of Ang II receptor subtypes on myocytes and nonmyocyte cells. A last but no less

TABLE 1. Characteristics of Subjects Investigated

	Control Subjects n=15	NF Hearts n=7	NYHA Class			
			I n=15	II n=14	III n=24	IV n=23
Age, years	60±10	44±5*	51±17	59±9	59±7	59±10
Sex (M/F)	13/2	5/2	10/5	13/1	21/3	17/6
Body surface area, m²	1.89±0.10	1.86±0.18	1.88±0.19	1.80±0.17	1.85±0.21	1.86±0.20
Treatments, No.						
Digitalis	0	0	0	8	14	16
Furosemide	0	0	0	5	22	23
ACE inhibitors	0	0	8	11	24	23
Beta blockers	0	0	2	2	3	3
Etiology (ICM/DCM)	...	...	8/7	8/6	13/11	11/12
Left ventricular end-diastolic diameter index, mm/m²	26.5±1.3	26.4±3.1	32.2±2.8†	32.7±2.8†	40.2±7.6†	42.4±5.6†
Left ventricular mass index, g/m²	109±13	104±18	175±39†	180±44†	234±65†	251±37†
Ejection fraction, %	65±6	61±3.9	54±11†	44±11†	24±13†	19±5†
Velocity of circumferential fiber shortening, s⁻¹	1.16±0.17	1.09±0.23	1.00±0.26	0.78±0.22†	0.53±0.09†	0.51±0.13†
End-systolic stress, kdyn/cm²	70±9	...	99±18†	115±32†	196±57†	212±51†
End-diastolic stress, kdyn/cm²	14±3	...	22±5†	32±12†	64±18†	74±10†
Cardiac index, L/m²×min⁻¹	3.23±0.49	...	2.80±0.26*	2.61±0.34†	2.08±0.46†	1.91±0.41†
Left ventricular end-diastolic pressure, mm Hg	8.3±1.5	...	13.6±4.1†	19.4±7.3†	27.1±6.6†	29.9±4.8†
Mean pulmonary artery pressure, mm Hg	11±3	...	14±4	20±6†	32±12†	36±15†
Pulmonary capillary wedge pressure, mm Hg	7±3	...	8±4	14±4†	21±9†	23±10†
PRA, ng/mL×hour⁻¹	0.82±0.28	...	1.09±0.81	1.63±1.37*	3.50±1.35†	4.41±0.79†

*$P<0.05$, †$P<0.01$ vs control subjects.

important question is the functional significance of cardiac Ang II formation in myocardial hypertrophy and HF. Although Ang II has been most frequently associated with both experimental and human hypertrophy,[12–14] the precise role of Ang II in cardiac hypertrophy remains elusive,[15,16] because cardiac hypertrophy may develop in the presence of Ang II generation inhibitors or AT_1 blockade.[17,18] Moreover, human compensatory hypertrophy is associated with increased cardiac formation of insulin-like growth factor-I (IGF-I) in volume overload and endothelin (ET)-1 and IGF-I in pressure overload, but not with cardiac Ang II.[14]

Thus, the functional significance of ACE mRNA and increased ACE activity found in expanded hearts remains to be clarified. Therefore, the present study was planned with the following objectives: (1) to investigate whether and when cardiac Ang II formation increases during the clinical course of HF and its relationship with left ventricular function, (2) to identify and examine the distribution of Ang II receptor subtypes on myocytes and nonmyocyte cells from failing and nonfailing (NF) myocardium, and (3) to study the relationship between Ang II and the capacity of myocytes for producing the hypertrophying growth factors IGF-I and ET-1.

Materials and Methods

We investigated 76 patients with HF due to dilated cardiomyopathy (DCM, n=36) or to ischemic cardiomyopathy (ICM, n=40). Diag-

nosis of DCM or ICM was based on clinical and echocardiographic examination, cardiac catheterization, and coronary angiography. HF patients were classified into functional groups according to the New York Heart Association (NYHA) classification (Table 1).

Patients with a recent history (<6 months) of myocardial infarction, a history of hypertension, echocardiographic evidence of valve or congenital heart disease, or an inability to suspend ACE inhibitors and diuretic treatment for 3 days were not considered for the study.

The control group was made up of 15 normotensive patients with atypical chest pain in whom angiography and routine diagnostic procedures did not reveal any abnormalities.

Cardiac specimens were obtained from 9 ICM and 13 DCM patients in NYHA classes III (n=10) and IV (n=12), who underwent cardiac transplantation, and 7 deceased donors with no history or signs of heart disease, whose hearts could not be transplanted because of surgical reasons or blood group incompatibility (NF hearts) (Table 1).

The protocol of this study complies with the principles of the Helsinki declaration. All patients gave their informed written consent to participate and to have their heart and blood samples used for the study. Echocardiographic and hemodynamic measurements were performed prospectively, as previously described.[14]

Cardiac formation of endogenous angiotensins was estimated as the aorta-coronary sinus gradient.[14] Ang I and Ang II plasma concentrations were measured by RIA after HPLC separation as previously described.[14] In 16 patients and 5 control subjects, the study of ¹²⁵I-labeled Ang I (¹²⁵I-Ang I) kinetics was simultaneously performed.[14]

Cardiomyocytes were isolated and membrane suspension was prepared from a noninfarcted portion of left ventricle free wall.[19] All
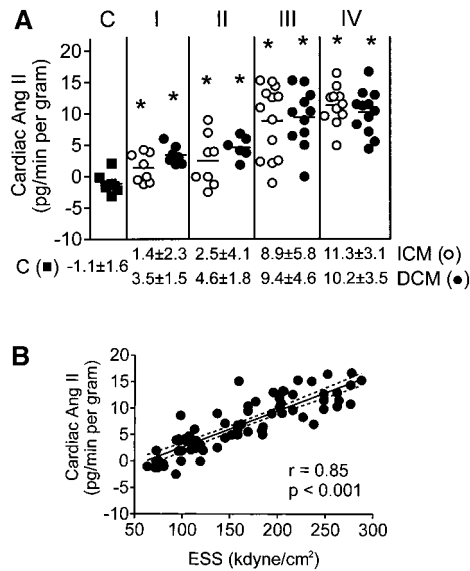
Figure 1. A, Ang II aorta–coronary sinus gradient indexed by coronary blood flow and cardiac mass (cardiac Ang II formation) in control subjects (■) and HF patients (○ indicates ICM; ●, DCM). Values under columns are mean±SD. *$P<0.05$ vs control subjects. B, Relationship between cardiac Ang II formation and ESS in HF patients.

studies were performed on freshly isolated cells. Binding studies were performed at equilibrium using ^{125}I-Ang II (100 pmol/L, 2000 Ci/mmol, Amersham) and selective AT_1 (Valsartan, a kind gift of Dr V. Abbruscato, Novartis, Italy) or AT_2 (PD-123319) antagonists. mRNAs for renin, chymase, angiotensinogen (AGTN), ACE, AT_1, and AT_2 were quantified by reverse transcriptase–polymerase chain reaction (RT-PCR) using specific primers with GAPDH as internal standard[14] and were expressed as the percentage of the values obtained in NF hearts.[14] The in situ hybridization procedure was performed using specific cDNA photobiotin-labeled (Vector) probes.[14] Myocardial cell types were identified by immunohistochemical methods using specific monoclonal antibodies. The presence of Ang II in myocardium was detected with the immunohistochemical method, using anti–Ang II rabbit antiserum (RIN 7002, Peninsula Laboratory, Inc). The stimulating effects of Ang II (10 nmol/L) on ET-1, IGF-I, and platelet-derived growth factor (PDGF) mRNA expression and peptide release by myocytes in vitro (100 000

myocytes/mL) were investigated in myocytes isolated from NF (n=4) and HF hearts (5 DCM and 4 ICM).

Data are mean±SD. Comparisons between groups were performed using 1-way ANOVA. For multivariate reevaluation of univariate correlations, the following were entered in a stepwise multiple regression analysis as independent variables, considering cardiac Ang II formation as a dependent variable: left ventricular end-diastolic diameter index, relative wall thickness, left ventricular mass index, left ventricular end-systolic volume index (LVESVI), left ventricular end-diastolic volume index (LVEDVI), left ventricular ejection fraction (LVEF), mean midwall velocity of circumferential fiber shortening (Vcf), end-systolic stress (ESS), end-diastolic stress, left ventricular end-diastolic pressure, Vcf/ESS ratio, Vcf/LVEDVI ratio, ESS/LVESVI ratio, and plasma renin activity (PRA).

An expanded Materials and Methods section can be found in an online data supplement available at http://www.circresaha.org.

Results

Cardiac Ang II Formation and Ventricular Function

Cardiac production of Ang II, as expressed by the mean aorta-coronary sinus concentration gradient, was already mildly but significantly increased in NYHA class I patients and further increased with the worsening of the functional class, with no differences being found between DCM and ICM patients (Figure 1A).

The ^{125}I-Ang I kinetics parameters are reported in Table 2. Neither extraction of both radiolabeled angiotensins nor the ^{125}I-Ang I–to–^{125}I-Ang II conversion rate differed between patients and control subjects (Table 2). Both Ang I and Ang II formation by cardiac tissues were on average significantly ($P<0.01$) higher in patients with mild HF (NYHA classes I and II) than in control subjects and further increased in patients with severe HF (NYHA classes III and IV; $P<0.01$). The increased Ang I de novo formation in patients with mild HF was mainly attributable to cardiac tissues, given that Ang I formed by PRA during the transcardiac passage of blood was not significantly different from that of control subjects (Table 2). In patients with severe HF, the total amount of Ang I formed was further increased versus mild HF with an increase in the contribution also by PRA (Table 2). Cardiac Ang II forma-

TABLE 2. ^{125}I-Ang I and ^{125}I-Ang II Concentrations at Steady State During ^{125}I-Ang I Infusion and Kinetics Parameters

Kinetics Parameters	Control Subjects n=5	Mild HF n=9	Severe HF n=7
^{125}I-Ang I extraction, %	35.2±3.8	34.2±4.1	36.0±5.7
^{125}I-Ang I to ^{125}I-Ang II conversion rate, %	24.3±4.7	25.1±4.9	24.9±5.2
Ang I by PRA, pg/mL	1.1±0.4	1.6±0.7	5.6±2.2*†
Ang I by cardiac tissues, pg/mL	2.5±1.3	6.2±3.9*	7.7±5.8*†
Total amount of Ang I formed, pg/mL	3.6±1.5	7.8±6.9*	13.5±6.4*†
^{125}I-Ang II extraction, %	26.8±2.8	27.9±1.9	27.8±2.2
Ang II by arterially delivered Ang I, pg/mL	1.1±0.6	1.2±0.7	1.4±0.8
Ang II by cardiac tissues, pg/mL	0.9±0.3	5.8±4.9*	10.3±5.9*†
Total amount of Ang II formed, pg/mL	2.1±0.8	7.2±3.4*	12.7±6.4*†
PRA, ng/mL×hour^{-1}	0.8±0.3	1.3±1.1*	4.0±0.6*†

Data are mean±SD.
*$P<0.05$ vs control subjects.
†$P<0.05$ vs mild HF.

TABLE 3. Univariate and Stepwise Multiple Regression Between Ang II Cardiac Formation and Hemodynamic and Echocardiographic Parameters

	r	P
ESS, kdyn/cm^2	0.85*	<0.001
Ejection fraction, %	−0.77	<0.001
Vcf/ESS	−0.75	<0.001
LVESVI, mm/m^2	0.74	<0.001
Vcf, s^{-1}	−0.72	<0.001
Relative wall thickness, %	−0.72	<0.001
Vcf/LVEDVI	−0.72	<0.001
EDS, kdyn/cm^2	0.64	<0.001
LVEDDI, mm/m^2	0.65	<0.001
PRA, ng/mL×hour^{-1}	0.63	<0.001
LVEDVI, mm/m^2	0.62	<0.001
LVMI, g/m^2	0.55	<0.001
ESS/LVESVI	−0.49	<0.001
LVEDP, mm Hg	0.37	<0.01

Vcf indicates velocity of circumferential fiber shortening; LVEDDI, left ventricular end-diastolic diameter index; LVMI, left ventricular mass index; and LVEDP, left ventricular end-diastolic pressure.

*Independent variable at stepwise regression.

tion measured by ^{125}I-Ang I kinetics was highly correlated with the aorta-coronary sinus gradient ($r=0.89$, $P<0.001$).

Ang II formation expressed by the aorta coronary sinus gradient was usually increased in patients with reduced LVEF, but it was found to be enhanced in a proportion of patients (18 of 76, 23%) with only mildly reduced LVEF (≥40%), provided that ESS was increased. Indeed, univariate regression analysis showed that Ang II formation was negatively correlated with left ventricular ejection fraction (LVEF, $r=-0.77$, $P<0.001$) and with the various indexes of ventricular contractility (Vcf, $r=-0.72$, $P<0.001$; Vcf/ESS, $r=-0.75$, $P<0.001$; Vcf/LVEDVI, $r=-0.72$, $P<0.001$; and ESS/LVESVI, $r=-0.49$, $P<0.001$). Conversely, it was positively correlated with ventricular systolic volume (LVESVI, $r=0.74$, $P<0.001$) and diastolic volume (LVEDVI, $r=0.62$, $P<0.001$). The multivariate stepwise analysis revealed that

ESS was the most predictive independent variable for Ang II formation ($r=0.85$, $P<0.001$) (Table 3, Figure 1B).

mRNA Expression of the Cardiac RAS Components

RT-PCR Assay

AGTN, ACE, and chymase genes were expressed in the NF hearts, whereas renin mRNA was not detectable (Figure 2). AGTN and ACE mRNA levels were higher in failing than in NF hearts, without any significant differences between ICM and DCM hearts (Figure 2). Conversely, chymase mRNA expression did not significantly differ in failing and NF hearts (Figure 2).

Messengers for AT$_1$ and AT$_2$ were expressed in all myocardial specimens. The AT$_1$ mRNA levels were lower in failing than in NF hearts ($P<0.01$) (Figure 2).

In Situ Hybridization

Negative and positive controls for in situ hybridization showed that the hybridization signal was specific for mRNA and that the mRNA in the hearts was intact). In NF hearts, mRNA for AGTN and ACE was expressed only in trace amounts. In the specimens from HF hearts, mRNA expression for AGTN and ACE was notably enhanced and was expressed almost exclusively on nonmyocyte cells (Figure 3).

Immunohistochemical Localization of Ang II

In NF hearts no immunostaining for Ang II was detected. In HF hearts immunostaining for Ang II was detected in both myocytes and interstitial cells (Figure 3).

Ang II Binding in NF and Failing Hearts

The density, affinity, and relative proportion of AT$_1$ to AT$_2$ subtypes in membranes and isolated myocytes are reported in Table 4.

In membranes from NF hearts, the proportion of AT$_1$ to AT$_2$ subtypes was 62:38 (Table 4). Total Ang II receptor and AT$_1$ subtype densities were significantly reduced in failing hearts as compared with NF hearts, with no significant differences between ICM and DCM hearts (Table 4).
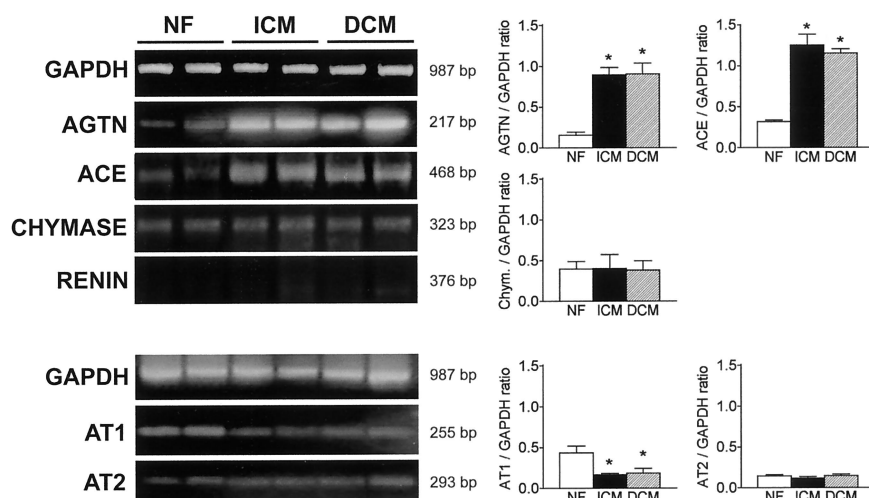

Figure 2. Left, Expression of mRNAs for AGTN, ACE, chymase, AT$_1$, AT$_2$, and GAPDH and lack of renin mRNA expression in ventricular homogenates from NF and failing hearts. Shown are representative RT-PCR experiments. Right, Bar graphs showing changes in densitometric ratio to GAPDH.
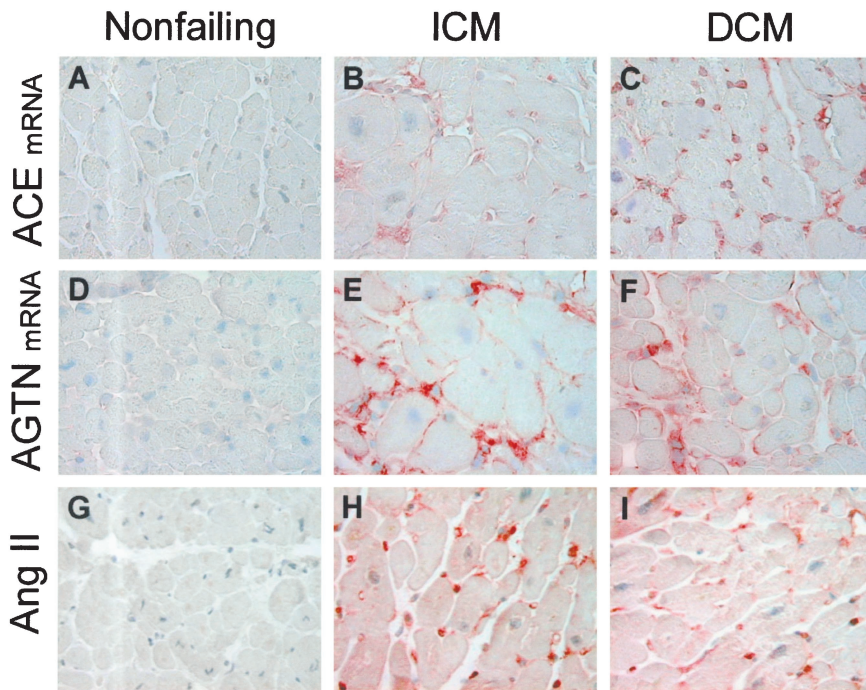
Figure 3. A through F, In situ hybridization for ACE (A, B, and C) and AGTN (D, E, and F) mRNAs in left ventricular sections from NF (A and D), ICM (B and E), and DCM (C and F) hearts. Positive mRNA signal is revealed by red-brown staining. In NF hearts, hybridization signals are almost absent. In failing hearts, mRNAs are detectable mainly in interstitial cells. G through I, Immunostaining for Ang II in left ventricular sections from NF (G), ICM (H), and DCM (I) hearts. Positive signal revealed by red-brown staining. In NF hearts no immunostaining for Ang II was detected. In failing hearts immunostaining for Ang II was detectable in both interstitial cells and myocytes. A through I, Magnification ×400.

Myocytes isolated from the left ventricle of NF hearts showed a prevalence of the AT$_1$ subtype (AT$_1$:AT$_2$=79:21). AT$_1$ and AT$_2$ density and affinity were unchanged in myocytes from failing hearts as compared with NF hearts (Table 4).

Effects of Ang II Stimulation on PreproET-1 (ppET-1), IGF-I, and PDGF Synthesis

Ventricular cardiomyocytes from NF hearts expressed very low levels of ppET-1, IGF-I, and PDGF mRNAs at quiescent state (Figure 4). Stimulation of myocytes with Ang II (10 nmol/L) did not change GAPDH gene expression (Figure 4) but induced a marked increase in ppET-1, IGF-I, PDGF-A, and PDGF-B mRNAs (Figure 4).

In cardiomyocytes from NF, ICM, or DCM hearts, Ang II failed to induce ppET-1 and IGF-I gene expression, whereas PDGF-B and PDGF-A gene expression was comparable among groups (Figure 4).

TABLE 4. Characterization of Ang II Receptors on Cardiomyocytes and Cardiac Membranes

	NF	ICM	DCM
Cardiomyocytes			
B$_{max}$ total, fmol/mg	0.57±0.21	0.55±0.10	0.60±0.11
K$_d$, nmol/L	0.19±0.08	0.21±0.06	0.23±0.10
AT$_1$:AT$_2$	79:21	76:24	73:27
B$_{max}$ AT$_1$, fmol/mg	0.45±0.17	0.41±0.07	0.44±0.08
B$_{max}$ AT$_2$, fmol/mg	0.12±0.04	0.14±0.04	0.16±0.05
Cardiac membranes			
B$_{max}$ total, fmol/mg	5.1±0.7	3.6±1.1*	3.4±0.9*
K$_d$, nmol/L	0.27±0.08	0.26±0.05	0.25±0.6
AT$_1$:AT$_2$	62:38	58:42	47:53
B$_{max}$ AT$_1$, fmol/mg	3.1±0.5	2.1±0.5*	1.6±0.5*
B$_{max}$ AT$_2$, fmol/mg	1.9±0.4	1.5±0.5	1.8±0.4

*$P<0.05$ vs control subjects.

The assay of big ET-1, IGF-I, and PDGF-AB in the conditioned media from stimulated cardiomyocytes confirmed at the peptide level the selective impairment of ET-1 and IGF-I response to Ang II stimulation (Figure 4).

Discussion

The major findings of this study may be summarized as follows: (1) the progression of HF is associated with a progressive increase in cardiac Ang II formation, regardless of the etiology of the HF and with a strong correlation with the increasing ESS; (2) the density and relative proportion of AT$_1$ and AT$_2$ on isolated HF myocytes are not significantly different from those on NF myocytes; and (3) Ang II stimulation does not induce HF myocytes to synthesize ET-1 and IGF-I.

Increased Ang II Formation and Cardiac Function

Although the gene overexpression of ACE in dilated cardiomyopathy was previously reported,[4] the finding of a high aorta–coronary sinus concentration gradient of both endogenous angiotensins and the kinetics study of ^{125}I-Ang I in the present work demonstrate an increased cardiac Ang II formation in HF. Upregulation of ACE and AGTN mRNAs and the immunohistochemical demonstration of Ang II in failing hearts corroborate this finding. It is worth stressing that the increased Ang II generation is independent of the HF etiology. In failing hearts, Ang II was detectable at immunohistochemistry both in myocytes and interstitial cells, whereas AGTN and ACE mRNAs were expressed predominantly in nonmyocytes. These findings may not be conflictual because in situ hybridization may be unable to reveal a low increase in mRNA expression and Ang II is uptaken by AT$_1$ and via endocytosis rapidly accumulated in myocytes.[20] The enhanced Ang II generation is mainly due to the de novo Ang I formation by cardiac tissues, despite the fact that RT-PCR did not reveal any expression of renin mRNA in either the NF
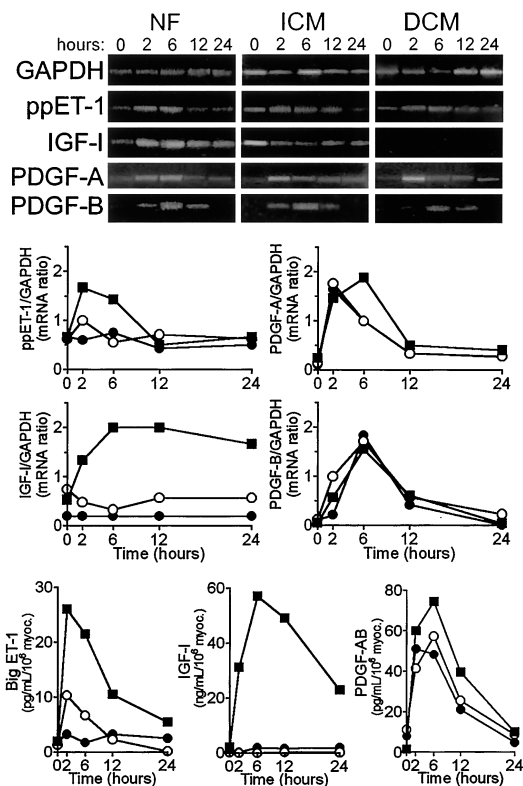
Figure 4. Top, Expression of GAPDH, ppET-1, IGF-I, PDGF-A, and PDGF-B by isolated ventricular cardiomyocytes after Ang II stimulation (10 nmol/L); representative RT-PCR experiments are shown. Middle, Densitometric growth factor/GAPDH mRNA ratio. Bottom: Release of big ET-1, IGF-I and PDGF-AB in the conditioned media from Ang II–stimulated cardiomyocytes. Data were normalized using the peptides released by unstimulated cells (blank subtracted). In middle and bottom panels, ■ indicates NF; ○, ICM; and ●, DCM.

or the failing hearts (Figure 2). However, the presence of renin mRNA and its overexpression in cardiac tissues from HF patients is not indispensable for Ang I formation, because there is wide evidence that renin may be uptaken from plasma by various renin binding proteins[21–23] and bound to subendothelium and endothelial cells.[6,21] There is also evidence that cardiac-bound renin may use the plasma AGTN present in cardiac extracellular fluid in addition to locally synthesized AGTN to generate Ang II.[6] Therefore, the finding of increased cardiac Ang I does not in any way contradict the finding that renin mRNA was not detectable in the HF hearts.

ACE mRNA levels in myocardium from failing hearts were higher than in NF hearts. In contrast, mRNA for chymase, a chymostatin-sensitive serine-proteinase,[7,8] was expressed only in traces, without any significant differences between NF and failing hearts. Thus, the present results suggest that in failing hearts Ang II generation mainly occurs through an ACE-dependent pathway.

Cardiac renin and plasma renin have been found to be in a diffusional steady state in rats,[24] and a positive correlation has been reported between renin plasma levels and the renin concentration in end-stage hearts from patients with DCM.[6] In this study as well, cardiac Ang II formation was correlated with PRA values in patients with severe HF (NYHA classes

III and IV; $r=0.47$, $P<0.001$) but not in those with mild HF (NYHA classes I and II; $r=0.32$, NS), the majority of whom had increased Ang II formation by cardiac tissues, although the PRA did not differ from that of control subjects. Likewise, multiple-regression analysis did not select PRA as a predictive independent variable for Ang II formation. These findings suggest that local cardiac factor(s) are preeminent in the activation of cardiac RAS in HF, even if important elevation in PRA contributes to the cardiac Ang II formation. Independently of the adjunctive role of PRA, the cardiac generation of both angiotensins progressively increases in relation with the impairment of cardiac function. The stepwise regression analysis revealed the ESS as the only variable independently correlated with Ang II formation ($r=0.85$). That the increase in ESS may play a major role in the upregulation of cardiac RAS is also supported by several studies that have shown that stretch induces Ang II formation both by isolated myocytes[25] and by beating hearts.[26,27] In patients with cardiac hypertrophy due to aortic valve disease, cardiac Ang II formation was again enhanced only in the patients with high ESS (>90 kdyn/cm^2), regardless of the type of overload (pressure or volume).[14] The close relationship between increased cardiac Ang II formation and high ESS values is not surprising, because ESS is a sensitive indicator of decompensation of dilated cardiomyopathy. Thus, clinical and experimental results suggest that ESS is a causative factor for the increase in Ang II formation in human hearts, probably stimulating the upregulation of both ACE and AGTN genes and facilitating the diffusion of renin from plasma to cardiac tissues. Cardiac Ang II production was negatively correlated with myocardial contractility as expressed not only by Vcf but also by the ESS/LVESVI ratio, which is a sensitive contractility index independent of ventricular size. However, ESS was the strongest predictor of Ang II production, thus indicating that a mechanical factor rather than the loss of contractility affects cardiac Ang II production.

Myocyte and Membrane Receptor Binding Sites

Although the presence of AT$_1$ was demonstrated using in situ RT-PCR in both myocytes and interstitial cells,[9] the density of Ang II binding sites both in failing and NF hearts has been studied only on cardiac homogenates. The results indicate a general tendency of AT$_1$ to decrease in failing hearts, with no changes in AT$_2$.[9,10] In contrast with these studies, Tsutsumi et al[11] reported that AT$_2$ binding sites and mRNA expression were increased in patients with dilated cardiomyopathy. In the present study, binding sites for Ang II receptors were separately investigated in isolated myocytes and in myocardial membranes. The results showed notable differences between myocytes and membranes from NF hearts in the distribution and relative proportion of the Ang II receptor subtypes. Ang II binding sites on myocytes from NF hearts were ≈10% of the binding sites detected on ventricular membranes, with a clear predominance of AT$_1$s, which were ≈4 times more numerous than AT$_2$. Most importantly, whereas binding site density for AT$_1$ was lower on heart membranes from failing than from NF hearts, the density of both AT$_1$ and AT$_2$ on isolated myocytes from HF hearts did

not significantly differ from that of NF hearts. Thus, for the first time we demonstrated that AT_1 density is decreased on cardiac membranes but not on myocytes from failing hearts, and the myocytes may therefore remain in end-stage failing hearts as potential targets for Ang II–mediated effects. The downregulation of Ang II receptors on nonmyocyte cells appears to be related to the HF and not to a disease-specific mechanism, because no differences between DCM and ICM hearts were found in the receptor density on either myocytes or membranes. Thus, the pattern of Ang II receptors does differ from that of ET-1 binding sites, which were increased in ICM hearts but not in DCM ones, with a proportional increase in ET_A and ET_B receptors both on myocytes and membranes.[19]

The mechanisms responsible for the lack of downregulation of AT_1 on myocytes in comparison with nonmyocyte cells, notwithstanding the increased Ang II, have not yet been clarified. Mechanical stretching increases AGTN mRNA levels and upregulates the number of AT_1s and AT_2s in neonatal cultured cardiac myocytes,[28–30] and this effect is potentiated by Ang II.[29] Moreover, Ang II levels were found to be directly related to AT_1 promoter activity in pressure-overloaded myocytes.[31] The combination of stretching and Ang II might thus keep the receptor density on failing heart myocytes similar to that of control subjects; conversely, the lower stretching effect on the nonmyocyte cells in comparison with myocytes might result in a predominance of the downregulating activity by the enhanced Ang II. Another possibility is that the different regulation of AT_1 and AT_2 may depend on differences in the regulation mechanism(s). Indeed, a different molecular mechanism has been found to regulate Ang II receptor subtype expression in the rat heart in a cell- and subtype-specific manner.[32] A similar mechanism might differently regulate the gene expression of AT_1 subtype on myocytes and on nonmyocyte cells. However, specifically addressed studies are needed to investigate the different expression of Ang II receptor subtypes in the failing human heart.

Incapacity of the Failing Myocytes to Synthesize IGF-I and ET-1 and Role of Ang II in HF

The third major result in this study is the demonstration that Ang II induces IGF-I, ET-1, and PDGF-AB synthesis by human isolated NF myocytes and that failing myocytes are selectively unable to produce appreciable amounts of IGF-I and ET-1 in response to Ang II stimulation, notwithstanding the similar density and binding capacity of Ang II receptor subtypes. The preserved PDGF-AB generation by failing myocytes after Ang II stimulation suggests a specific impairment of the pathways leading to IGF-I and ET-1 formation rather than a general aspecific desensitization of the Ang II receptors. The mechanisms responsible for the incapacity of failing myocytes to produce IGF-I and ET-1 after Ang II stimulation remain to be investigated. Multiple signal-transduction pathways are activated in response to AT_1 stimulation,[33] and many steps intervene between receptor activation and the molecular response that increases myocyte IGF-I and ET-1 synthesis. Thus, the defective response of failing myocytes to Ang II stimulation may reside either in

intracellular signal transduction pathways or in transcription factors. Specific studies are, however, necessary to investigate this problem.

Cardiac IGF-I and ET-1 play a critical role in supporting cardiac adaptive response to hemodynamic overload. Human compensatory hypertrophy due to aortic valve disease is associated with an increased cardiac myocyte formation of IGF-I in volume overload and IGF-I and ET-1 in pressure overload.[14] Cardiac production of these growth factors is positively related to myocardial contractility.[14] The depression of contractility, the increase in ESS, and the progression toward noncompensatory hypertrophy and HF are associated with the decrease in IGF-I and ET-1 myocyte formation and with the increase in Ang II generation.[14] In experimental pressure or volume overload in pigs, aortic banding or the creation of an aorta-cava shunt are immediately followed by an increased cardiac Ang II formation (within 3 hours), leading to enhanced ET-1 and IGF-I generation by myocytes with recovery of contractility, ESS normalization, and return of Ang II formation to resting values within 12 hours.[34] It is important to highlight that Ang II, ET-1, and IGF-I are synthesized by myocytes, in contrast with the incapacity of human overloaded[14] or failing myocytes to synthesize not only ET-1 and IGF-I, but also Ang II itself, which appears to be essentially formed by nonmyocyte cells (Reference 14 and the present study). Thus, the increased cardiac Ang II formation triggered by the increase in the ESS operates as an inducing factor for the formation of IGF-I and ET-1 (and perhaps other growth factors) by myocytes. In noncompensatory hypertrophy and HF, myocytes are unable to respond to the stimulating activity of Ang II. Because AT_1s on myocytes are not downregulated in failing hearts, the long-lasting excessive Ang II formation can mediate detrimental effects on overloaded or failing myocytes, including depression of contractility or impaired relaxation.[35] AT_1s also remain well represented on fibroblasts from failing hearts[36] with consequent interstitial extracellular matrix accumulation.[11] Moreover, experimental evidence suggests that local Ang II through AT_1 activates the transcription factor nuclear factor-κB,[37] thereby promoting overexpression of numerous genes, including various cytokines and adhesion molecules.

In conclusion, the present study demonstrates that early in the clinical course of HF, cardiac Ang II formation is increased, myocyte AT_1s are not downregulated, and myocytes are unable to synthesize IGF-I and ET-1 in response to Ang II stimulation.

References
1. Dostal DE, Baker KM. The cardiac renin angiotensin system: conceptual, or a regulator of cardiac function? *Circ Res*. 1999;85:643–650.
2. Neri Serneri GG, Boddi M, Coppo M, Chechi T, Zarone N, Moira M, Poggesi L, Margheri M, Simonetti I. Evidence for the existence of a functional cardiac renin-angiotensin system in humans. *Circulation*. 1996;94:1886–1893.
3. Wollert KC, Drexler H. The renin-angiotensin system and experimental heart failure. *Cardiovasc Res*. 1999;43:838–849.

4. Studer R, Reinecke H, Muller B, Holtz J, Just H, Drexler H. Increased angiotensin-I converting enzyme gene expression in the failing human heart: quantification by competitive RNA polymerase chain reaction. *J Clin Invest*. 1994;94:301–310.

5. Zisman LS, Asano K, Dutcher DL, Ferdensi A, Robertson AD, Jenkin M, Bush EW, Bohlmeyer T, Perryman MB, Bristow MR. Differential regulation of cardiac angiotensin converting enzyme binding sites and AT_1 receptor density in the failing human heart. *Circulation*. 1998;98: 1735–1741.

6. Danser AH, van Kesteren CA, Bax WA, Tavenier M, Derkx FH, Saxena PR, Schalekamp MA. Prorenin, renin, angiotensinogen, and angiotensin-converting enzyme in normal and failing human hearts: evidence for renin binding. *Circulation*. 1997;96:220–226.

7. Urata H, Healey B, Stewart RW, Bumpus FM, Husain A. Angiotensin II-forming pathways in normal and failing human harts. *Circ Res*. 1990; 66:883–890.

8. Wolny A, Clozel JP, Rein J, Mory P, Vogt P, Turino M, Kiowski W, Fischli W. Functional and biochemical analysis of angiotensin II-forming pathways in the human heart. *Circ Res*. 1997;80:219–227.

9. Asano K, Dutcher DL, Port JD, Minobe WA, Tremmel KD, Roden RL, Bohlmeyer TJ, Bush EW, Jenkin MJ, Abraham WT, Raynolds MV, Zisman LS, Perryman MB, Bristow MR. Selective downregulation of the angiotensin II AT_1-receptor subtype in failing human ventricular myocardium. *Circulation*. 1997;95:1193–1200.

10. Haywood GA, Gullestad L, Katsuya T, Hutchinson HG, Pratt RE, Horiuchi M, Fowler MB. AT_1 and AT_2 angiotensin receptor gene expression in human heart failure. *Circulation*. 1997;95:1201–1206.

11. Tsutsumi Y, Matsubara H, Ohkubo N, Mori Y, Nozawa Y, Murasawa S, Kijima K, Maruyama K, Masaki H, Moriguchi Y, Shibasaki Y, Kamihata H, Inada M, Iwasaka T. Angiotensin II type 2 receptor is upregulated in human heart with interstitial fibrosis, and cardiac fibroblasts are the major cell type for its expression. *Circ Res*. 1998;83:1035–1046.

12. Baker KM, Chernin MI, Wixson SK, Aceto JF. Renin-angiotensin system involvement in pressure-overload cardiac hypertrophy in rats. *Am J Physiol*. 1990;259:H324–H332.

13. Schunkert H, Dzau VJ, Tang SS, Hirsch AT, Apstein CS, Lorell BH. Increased rat cardiac angiotensin converting enzyme activity and mRNA expression in pressure overload left ventricular hypertrophy: effects on coronary resistance, contractility, and relaxation. *J Clin Invest*. 1990;86: 1913–1920.

14. Neri Serneri GG, Modesti PA, Boddi M, Cecioni I, Paniccia R, Coppo M, Galanti G, Simonetti I, Vanni S, Papa L, Bandinelli B, Migliorini A, Modesti A, Maccherini M, Sani G, Toscano M. Cardiac growth factors in human hypertrophy: relations with myocardial contractility and wall stress. *Circ Res*. 1999;85:57–67.

15. Yamazaki T, Yazaki Y. Is there major involvement of the renin-angiotensin system in cardiac hypertrophy? *Circ Res*. 1997;81:639–642.

16. Cooper G IV. Basic determinants of myocardial hypertrophy: a review of molecular mechanisms. *Annu Rev Med*. 1997;48:13–23.

17. Koide M, Carabello BA, Conrad CC, Buckley JM, DeFreyte G, Barnes M, Tomanek RJ, Wei CC, Dell'Italia LJ, Cooper G IV, Zile MR. Hypertrophic response to hemodynamic overload: role of load vs renin-angiotensin system activation. *Am J Physiol*. 1999;276:H350–H358.

18. Harada K, Komuro I, Shiojima I, Hayashi D, Kudoh S, Mizuno T, Kijima K, Matsubara H, Sugaya T, Murakami K, Yazaki Y. Pressure overload induces cardiac hypertrophy in angiotensin II type 1A receptor knockout mice. *Circulation*. 1998;97:1952–1959.

19. Neri Serneri GG, Cecioni I, Vanni S, Paniccia R, Bandinelli B, Vetere A, Janming X, Bertolozzi I, Boddi M, Lisi GF, Sani G, Modesti PA. Selective upregulation of cardiac endothelin system in patients with ischemic but not idiopathic dilated cardiomyopathy: endothelin-1 system in the human failing heart. *Circ Res*. 2000;86:377–385.

20. Robertson AL, Khairallah PA. Angiotensin II: rapid localization in nuclei of smooth and cardiac muscle. *Science*. 1971;172:1138–1139.

21. Danser AH, van Kats JP, Admiraal PJ, Derkx FH, Lamers JM, Verdouw PD, Saxena PR, Schalekamp MA. Cardiac renin and angiotensins: uptake from plasma versus in situ synthesis. *Hypertension*. 1994;24:37–48.

22. Maru I, Ohta Y, Murata K, Tsukada Y. Molecular cloning and identification of N-acyl-D-glucosamine 2-epimerase from porcine kidney as a renin-binding protein. *J Biol Chem*. 1996;271:16294–16299.

23. Sealey JE, Catanzaro DF, Lavin TN, Gahnem F, Pitarresi T, Hu LF, Laragh JH. Specific prorenin/renin binding (ProBP). Identification and characterization of a novel membrane site. *Am J Hypertens*. 1996;9: 491–502.

24. Heller LJ, Opsahl JA, Wernsing SE, Saxena R, Katz SA. Myocardial and plasma renin-angiotensinogen dynamics during pressure-induced cardiac hypertrophy. *Am J Physiol*. 1998;274:R849–R856.

25. Sadoshima J, Xu Y, Slayter HS, Izumo S. Autocrine release of angiotensin II mediates stretch-induced hypertrophy of cardiac myocytes in vitro. *Cell*. 1993;75:977–984.

26. Lee YA, Liang CS, Lee MA, Lindpaintner K. Local stress, not systemic factors, regulate gene expression of the cardiac renin-angiotensin system in vivo: a comprehensive study of all its components in the dog. *Proc Natl Acad Sci U S A*. 1996;93:11035–11040.

27. Horban A, Kolbeck-Ruhmkorff C, Zimmer HG. Correlation between function and proto-oncogene expression in isolated working rat hearts under various overload conditions. *J Mol Cell Cardiol*. 1997;29: 2903–2914.

28. Kijima K, Matsubara H, Murasawa S, Maruyama K, Mori Y, Ohkubo N, Komuro I, Yazaki Y, Iwasaka T, Inada M. Mechanical stretch induces enhanced expression of angiotensin II receptor subtypes in neonatal rat cardiac myocytes. *Circ Res*. 1996;79:887–897.

29. Tamura K, Umemura S, Nyui N, Hibi K, Ishigami T, Kihara M, Toya Y, Ishii M. Activation of angiotensinogen gene in cardiac myocytes by angiotensin II and mechanical stretch. *Am J Physiol*. 1998;275:R1–R9.

30. Malhotra R, Sadoshima J, Brosius FC III, Izumo S. Mechanical stretch and angiotensin II differentially upregulate the renin-angiotensin system in cardiac myocytes in vitro. *Circ Res*. 1999;85:137–146.

31. Herzig TC, Jobe SM, Aoki H, Molkentin JD, Cowley AW Jr, Izumo S, Markham BE. Angiotensin II type1a receptor gene expression in the heart: AP-1 and GATA-4 participate in the response to pressure overload. *Proc Natl Acad Sci U S A*. 1997;94:7543–7548.

32. Matsubara H, Kanasaki M, Murasawa S, Tsukaguchi Y, Nio Y, Inada M. Differential gene expression and regulation of angiotensin II receptor subtypes in rat cardiac fibroblasts and cardiomyocytes in culture. *J Clin Invest*. 1994;93:1592–1601.

33. Sadoshima J. Versatility of the angiotensin II type 1 receptor. *Circ Res*. 1998;82:1352–1355.

34. Modesti PA, Vanni S, Bertolozzi I, Cecioni I, Polidori G, Paniccia R, Bandinelli B, Perna A, Liguori P, Boddi M, Galanti G, Neri Serneri GG. Early sequence of cardiac adaptations and growth factor formation in pressure- and volume-overload hypertrophy. *Am J Physiol*. 2000;279: H976–H985.

35. Cheng CP, Suzuki M, Ohte N, Ohno M, Wang ZM, Little WC. Altered ventricular and myocyte response to angiotensin II in pacing-induced heart failure. *Circ Res*. 1996;78:880–892.

36. Kawano H, Do YS, Kawano Y, Starnes V, Barr M, Law RE, Hsueh WA. Angiotensin II has multiple profibrotic effects in human cardiac fibroblasts. *Circulation*. 2000;101:1130–1137.

37. Ruiz-Ortega M, Lorenzo O, Ruperez M, Konig S, Wittig B, Egido J. Angiotensin II activates nuclear transcription factor κB through AT_1 and AT_2 in vascular smooth muscle cells: molecular mechanisms. *Circ Res*. 2000;86:1266–1272.