Impaired angiotensin II–extracellular signal-regulated kinase signaling in failing human ventricular myocytes

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Angiotensin II was reported to induce insulin-like growth factor-I and endothelin-1 gene expression and peptide release by ventricular cardiomyocytes. However, the progression from cardiac hypertrophy to failure in humans is characterized by a reduced myocyte expression of insulin-like growth factor-I and endothelin-1, notwithstanding the enhanced cardiac generation of angiotensin II. In the present study we investigated the functional status of the signaling pathways responsible for angiotensin II-induced endothelin-1 and insulin-like growth factor-I formation in human ventricular myocytes isolated from patients with dilated ($n = 19$) or ischemic ($n = 14$) cardiomyopathy and nonfailing donor hearts ($n = 6$).

In human nonfailing ventricular myocytes, angiotensin II (100 nmol/l) induced insulin-like growth factor-I and endothelin-1 gene expression, and peptide release was mediated by extracellular signal-regulated kinase activation and inhibited by extracellular signal-regulated kinase antagonism (PD98059, 30 μmol/l), endothelin-1 formation being partially reduced also by c-Jun N-terminal kinase inhibition (SP600125, 10 μmol/l); insulin-like growth factor-I and endothelin-1 formations were unaffected by the inhibition of p38 mitogen-activated protein kinase (SB203580, 10 μmol/l) and Janus tyrosine kinase 2 (AG490, 10 μmol/l). In failing myocytes, angiotensin II failed to induce insulin-like growth factor-I and endothelin-1 formation; angiotensin II-induced extracellular signal-regulated kinase activation was significantly impaired (−88% vs. controls) although c-Jun NH_2-terminal kinase activation was preserved. The impaired extracellular signal-regulated kinase phosphorylation in failing myocytes was associated with increased myocyte levels of mitogen-activated protein kinase phosphatases.

Therefore, the altered growth factor production in failing myocytes is associated with a significant derangement in intracellular signaling. *J Hypertens* 26:2030–2039 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Abbreviations: Ang II, angiotensin II; AT1, angiotensin II type 1; AT2, angiotensin II type 2; BSA, body surface area; DCM, dilated cardiomyopathy; ERK, extracellular signal-regulated kinase; ESS, end systolic stress; ET-1, endothelin-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ICM, ischemic cardiomyopathy; IGF-I, insulin-like growth factor-1; IgG, immunoglobulin G; IP3, inositol 1,4,5-trisphosphate; JAK2, Janus tyrosine kinase 2; JNK, c-Jun N-terminal kinase; LVEDDI, left ventricular end diastolic diameter index; LVEDP, left ventricular end diastolic pressure; LVESDI, left ventricular end systolic diameter index; LVMI, left ventricular mass index; mAbs, monoclonal antibodies; MAPK, mitogen-activated protein kinase; MEM, minimal essential medium; MKP, mitogen-activated protein kinase phosphatase; MPAP, mean pulmonary arterial pressure; p-c-Jun, phosphorylated c-Jun; p-ERK, phosphorylated extracellular signal-regulated kinase; ppET-1, prepro endothelin-1; SAP, systolic aortic pressure; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Vcf, mean midwall velocity of circumferential fiber shortening

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Introduction

Angiotensin (Ang) II induces endothelin-1 (ET-1), and insulin-like growth factor-1 (IGF-I) formation in isolated ventricular myocytes. Early growth factor formation following the application of mechanical stimuli is also mediated by Ang II as suggested by the observation that the inhibition of type I receptors for Ang II inhibited early growth factor (ET-1 and angiotensinogen) overexpression in stretched myocytes *in vitro* [1] and in experimental models of acute cardiac overload *in vivo* [2,3]. However, in patients with aortic valve disease, the progression from compensatory hypertrophy to heart failure is characterized by reduced gene expression of ventricular myocytes and cardiac formation of insulin-like growth factor-1 (IGF-I) and ET-1 [4], notwithstanding a significant increase in cardiac Ang II generation [4]. The capability of myocytes to express IGF-I and ET-1 following Ang II stimulation is finally lost in ventricular myocytes isolated from failing explanted human hearts [5]. These data and the absence of differences in Ang II

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type 1 (AT1) and type 2 (AT2) receptor populations between nonfailing and failing human ventricular myocytes [5] might lead to the hypothesis that the progression to failure is characterized by an alteration of intracellular signaling pathways. Different studies investigated protein amounts and activity of the three branches of the mitogen-activated protein kinase (MAPK) family, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38MAPK, in whole homogenated human failing hearts [6,7]. In isolated human failing ventricular myocytes, the activation of the Ang II–Janus tyrosine kinase (JAK) 2 pathway was recently reported [8], but information regarding the Ang II–ERK signaling is lacking. This aspect might be relevant because ERK is known to regulate myocyte survival [9,10] and increases the expression of transcription factors, which recognize specific DNA region binding in the promoter of both IGF-I [11] and ET-1 [12] genes. IGF-I promotes myocyte formation and attenuates myocyte death after infarction [13]. Likewise, ET-1 is required for cardiomyocyte survival *in vivo* [14] and was reported to mediate the positive inotropic effect of Ang II [15]. MAPK activity is negatively regulated by dual-specificity MAPK phosphatases (MKPs) that are expressed in the heart and are induced by mitogen and stress stimuli [16]. Cardiac overexpression of MKP3 was recently reported to potently block ERK, predisposing the heart to decompensation and failure after long-term pressure overload [17].

The present study was therefore performed to investigate the functional status of Ang II–ERK signaling and of protein phosphatases (MKP1, MKP2, MKP3) in failing human myocytes.

Patients and methods

Study population

Patients with heart failure due to idiopathic dilated cardiomyopathy (DCM, $n = 19$) or ischemic cardiomyopathy (ICM, $n = 14$) belonging to New York Heart Association (NYHA) class III (DCM, $n = 11$ and ICM, $n = 8$) or class IV (DCM, $n = 8$ and ICM, $n = 6$) and scheduled to undergo cardiac transplantation were prospectively investigated. A recent history (less than 6 months) of myocardial infarction and echocardiographic evidence of valve or congenital heart disease were exclusion criteria. Diagnosis of DCM or ICM was based on clinical and echocardiographic examination, cardiac catheterization, and coronary angiography. Myocardial tissue was obtained from a noninfarcted portion of the left ventricular free wall during cardiac transplantation. Cardiac specimens were also obtained from the same cardiac region of six donors with no history or signs of heart disease and whose hearts could not be transplanted for noncardiac reasons (nonfailing hearts) (Table 1). The study protocol complies with the principles of the Helsinki declaration, was approved by our institution, and all patients gave their informed written consent to participate in the study. Echocardiographic and hemodynamic measurements were performed as previously described [4].

Table 1 Characteristics of individuals investigated

	NF ($n = 6$)	ICM ($n = 14$)	DCM ($n = 19$)
Age (years)	46 ± 7	$59 \pm 5^*$	$51 \pm 13^*$
Sex (M/F)	4/2	11/3	13/6
BSA (m^2)	1.87 ± 0.12	1.91 ± 0.14	1.88 ± 0.20
SAP (mmHg)	118 ± 8	111 ± 11	115 ± 9
LVEDDI (mm/m^2)	26 ± 3	$38 \pm 5^*$	$42 \pm 6^*$
LVESDI (mm/m^2)	22 ± 4	$32 \pm 5^*$	$36 \pm 5^*$
LVMI (g/m^2)	107 ± 15	$221 \pm 36^*$	$250 \pm 60^*$
EF (%)	63 ± 5	$22 \pm 8^*$	$19 \pm 7^*$
ESS ($Kdyne/cm^2$)	70 ± 9	$181 \pm 36^*$	$195 \pm 15^*$
Vcf (s^{-1})	1.16 ± 0.17	$0.52 \pm 0.2^*$	$0.48 \pm 0.17^*$
CI [$l/(m^2 \times min)$]		2.13 ± 0.48	1.95 ± 0.50
LVEDP (mmHg)		22 ± 7	26 ± 9
MPAP (mmHg)		33 ± 11	37 ± 12

BSA, body surface area; CI, cardiac index; DCM, dilated cardiomyopathy; EF, ejection fraction; ESS, end systolic stress; ICM, ischemic cardiomyopathy; LVEDDI, left ventricular end diastolic diameter index; LVEDP, left ventricular end diastolic pressure; LVESDI, left ventricular end systolic diameter index; LVMI, left ventricular mass index; MPAP, mean pulmonary arterial pressure; NF, nonfailing; SAP, systolic aortic pressure; Vcf, mean midwall velocity of circumferential fiber shortening. $^*P < 0.05$ vs. NF.

Myocyte isolation

All studies were performed on freshly isolated ventricular myocytes. After explantation, the heart was placed in cardioplegic solution and immediately transferred to the laboratory.

Myocytes were isolated with the enzymatic digestion method as previously described [5,8]. Briefly, a coronary artery branch was cannulated and perfused for 10 min with a calcium-free basic buffer (blood washout) composed of Jocklik modified minimal essential medium (MEM Joklik) supplemented with glutamine (Sigma G5763, Sigma Chemical Co., St. Louis, Missouri, USA) (0.3 g/l), taurine (Sigma T0625) (1.25 g/l), HEPES (Sigma H3375) (2.9 mmol/l), insulin (20 U/l), penicillin–streptomycin (Sigma P3539, 50 U/ml and 0.05 mg/ml, respectively) 5 ml/l, and $CaCl_2$ (7.5 μmol/l), pH 7.4. The basic buffer was previously leaked through filters of 0.2 μm pore size. Perfusion was then switched to collagenase solution, composed of 0.5 mg/ml of Worthington type II collagenase (100 units/ml) in basic buffer supplemented with $CaCl_2$ 30 μmol/l (20 ml/min for 20–25 min). The collagenase-perfused tissue was then minced and tissue was collected in tubes containing basic buffer supplemented with bovine serum albumin (0.5%), $CaCl_2$ (0.3 mmol/l), and taurine (10 mmol/l). Individual myocytes were then released from the tissue by mechanical agitation. The suspension was filtered through a sterile gauze to separate cells from tissue mass. The populations of cells were then washed using two complete cycles of low-speed centrifugation. The dispersed cells were finally preplated for 30 min to minimize fibroblast contamination. Typical preparations contained 60–70% rod-shaped, quiescent

Ca^{2+}-tolerant myocytes that had well defined, regular cross-striations and sarcomere patterns.

Staining of sarcomeric alpha-actinin and myosin was performed using antisarcomeric alpha-actinin monoclonal antibodies (mAbs) at a dilution of 1 : 800 and rabbit polyclonal antiskeletal myosin mAbs at a dilution of 1 : 200 (both from Sigma Chemical Co.), respectively. Samples were then washed in PBS/1% bovine serum albumin three times for 5 min each. Secondary detection was carried out by incubation for 30 min at room temperature with a 1 : 200 dilution of fluorescein-conjugated or rhodamine-conjugated, noncross-reactive, goat antimouse or antirabbit antibodies, respectively. According to their appearance under phase contrast microscopy and by immunocytochemical staining, nonmyocyte cells were found to account for less than 2% of the total cells [5,8].

Angiotensin II stimulation and measurement of insulin-like growth factor-I and endothelin-1

After isolation, myocytes (10^5 cell/ml) were suspended in Dulbecco's Modified Eagle's Medium supplemented with penicillin G (10 000 U/ml) and streptomycin (10 mg/ml) under an atmosphere of 95% air and 5% CO_2 at 37°C. Myocyte levels of AT1 and AT2 transcripts were quantified with reverse transcriptase-PCR, using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal standard [5].

To investigate the relative role of the two angiotensin receptor subtypes, myocytes were preincubated for 30 min with selective AT1 or AT2 receptor antagonists (valsartan, 1 μmol/l or PD123319, 1 μmol/l, respectively). To investigate the relative role of the different intracellular pathways in the Ang II-mediated activation of IGF-I and ET-1 gene expression and peptide release, nonfailing myocytes were preincubated (60 min) with inhibitors of ERK (PD98059, 30 μmol/l), JNK (SP600125, 10 μmol/l), p38 MAPK (SB203580, 10 μmol/l) and JAK2 (AG490, 10 μmol/l). Preliminary experiments using the trypan blue exclusion method had shown that no toxic effects were detected when the myocytes were incubated for as long as 6 h with the various inhibitors of intracellular signaling pathways.

Myocytes were then stimulated with Ang II (100 nmol/l) [5,18]. At baseline and after 2 h, the conditioned media and myocytes were separated by centrifugation and immediately frozen in liquid nitrogen.

IGF-I and preproET (ppET)-1 mRNA expression and peptides released in the conditioned media were measured with reverse transcriptase-PCR and radioimmunoassay (Peninsula Lab, San Carlos, California, USA) as previously described in detail [4,5].

Studies of the angiotensin II intracellular signaling pathways

After 5, 10, 20, 30, and 60 min of incubation under the conditions described above, the reaction samples were washed in ice-cold PBS and lysed in ice-cold lysis buffer (50 mmol/l Tris-HCl, 150 mmol/l NaCl, 1 mmol/l sodium orthovanadate, 100 mmol/l NaF, 2 mmol/l EGTA, 1% NP40, 1 mmol/l PMSF, pH 7.5) supplemented with Protease Inhibitor Cocktail (P8340, Sigma) and Phosphatase Inhibitor Cocktail (P5726, Sigma). Lysates were kept on ice and then sonicated four times for 5 s. After centrifugation, protein concentration in the supernatant fraction was assessed using Bradford's method.

Measurement of mitogen-activated protein kinase activity

Activated ERK dually phosphorylated on T202/Y204 was immunoprecipitated from lysate-stimulated cells and used to measure the incorporation of [γ-^{33}P]ATP into a specific p44/42 MAPK substrate (synthetic peptide, KRELVEPLTPAGEAPNQALLR) [19].

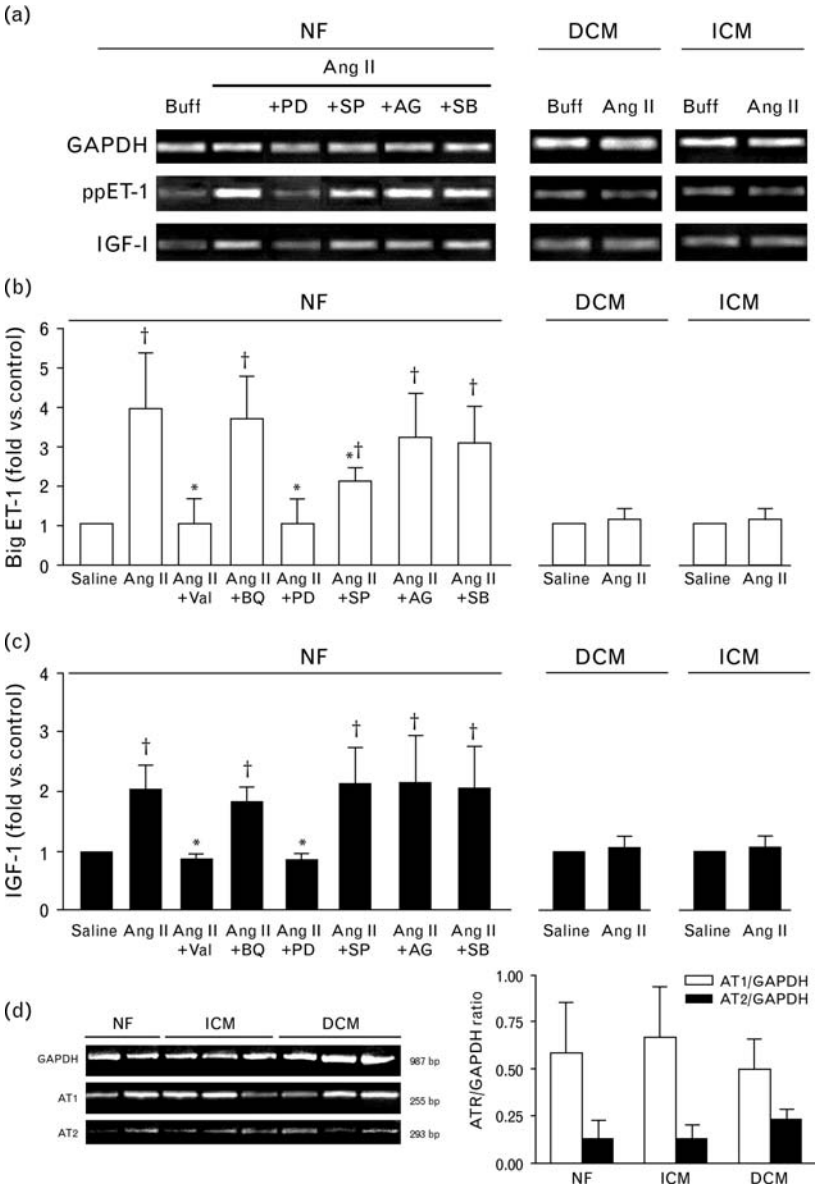
JNK activity was assayed using a nonradioactive method (Cell Signaling Technology, Beverley, Massachusetts, USA) [20]. Briefly, cell lysate (200 μg of protein) was incubated overnight at 4°C with 2 μg glutathione-*S*-transferase (GST)–c-Jun protein beads. Complexes were collected, washed, and resuspended in 50 μl kinase lysis buffer with 100 μmol/l ATP. Samples were incubated for 30 min at 30°C and the reaction was terminated with sample buffer. After separation with 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), western blot was performed using anti-phospho-c-Jun antibody.

Western blotting studies

Samples (25 μg proteins) were then separated with 8% SDS-PAGE. The gel was then transferred to nitrocellulose membrane (Hybond-ECL, Amersham Biosciences, Piscataway, New Jersey, USA) and blocked by incubation for 1 h at room temperature in tris-buffered saline with Tween-20 (TTBS, pH 7.4) and 5% skimmed milk powder.

Membranes were then incubated overnight at 4°C with polyclonal rabbit antihuman ERK, polyclonal rabbit antihuman phospho-ERK (Cell Signaling Technology, Beverly, Massachusetts, USA) [21], polyclonal rabbit antihuman MKP1 (sc-1199 Santa Cruz, Santa Cruz, California, USA), MKP2 (sc-10797 Santa Cruz), and MKP3 (sc-28902 Santa Cruz). The nitrocellulose membranes were then washed twice for 10 min with TTBS and incubated for 30 min with goat antirabbit or sheep antimouse immunoglobulin G (IgG) (as appropriate) horseradish peroxidase conjugate antibody (Amersham Biosciences). After extensive washing, the bound antibody was visualized using a chemioluminescent detection system.

Fig. 1



(a) Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ppET-1, and IGF-I genes by ventricular cardiomyocytes isolated from the hearts of donors (NF, $n=4$), and patients with dilated (DCM, $n=12$) or ischemic cardiomyopathy (ICM, $n=9$) after 2 h incubation with buffer (Buff) or Ang II (100 nmol/l). Effects of inhibitors of ERK (PD098059, 30 μmol/l), JNK (SP600125, 10 μmol/l), JAK2 (AG490, 10 μmol/l), and p38MAPK (SB203580, 10 μmol/l). Representative reverse transcriptase-PCR experiments. (b, c) Release of big ET-1 (b) and IGF-I (c) in the conditioned media after 2 h stimulation with Ang II (100 nmol/l) for myocytes isolated from controls ($n=6$), DCM ($n=19$), and ICM ($n=14$). Data were normalized using the peptides released by unstimulated cells. Effects of ET-1 antagonist (BQ123, 10 μmol/l), and inhibitors of ERK (PD098059, 30 μmol/l), JNK (SP600125, 10 μmol/l), JAK2 (AG490, 10 μmol/l), and p38MAPK (SB203580, 10 μmol/l). $^{\dagger}P<0.05$ vs. myocytes incubated with buffer (saline); $^{*}P<0.05$ vs. Ang II-stimulated myocytes. (d) Expression of mRNAs for GAPDH, AT1, and AT2 receptor subtypes in ventricular myocytes isolated from donors (NF) and failing (ICM, DCM) hearts. Left panel: representative reverse transcriptase-PCR experiments. Right graph: densitometric Ang II receptor/GAPDH (ATR/GAPDH) mRNA ratio (empty bars, AT1; filled bars, AT2) in myocytes isolated from NF ($n=4$), ICM ($n=12$), and DCM ($n=9$) hearts. Ang II, angiotensin II; AT1, angiotensin II type 1; AT2, angiotensin II type 2; DCM, dilated cardiomyopathy; ERK, extracellular signal-regulated kinase; ET-1, endothelin-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ICM, ischemic cardiomyopathy; IGF-I, insulin-like growth factor-1; JAK2, Janus tyrosine kinase 2; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NF, nonfailing; ppET-1, prepro endothelin-1.

Statistical analysis

Data are expressed as mean ± SD. Autoradiograms were analyzed using an image analyzer. Comparisons between groups were performed using one-way analysis of variance and student's *t* test, followed by the Tukey multiple-range comparison test, as appropriate. A *P* value of 0.05 was considered statistically significant.

Results

Signaling pathways involved in the angiotensin II-induced endothelin-1 and insulin-like growth factor-I production by human nonfailing myocytes

Ang II stimulation of nonfailing myocytes enhanced ET-1 and IGF-I gene expression (Fig. 1a) as well as peptide release in the incubation medium (Fig. 1b and c). The Ang II-induced increase in big ET-1 or IGF-I formation in nonfailing myocytes was mediated by the AT1 receptor as the response was selectively inhibited by valsartan (Fig. 1b and c). Conversely, the release of both big ET-1 and IGF-I was unaffected by the specific antagonist for the type A ET-1 receptor subtype (BQ123) (Fig. 1b and c), thus excluding an autocrine/paracrine effect of ET-1.

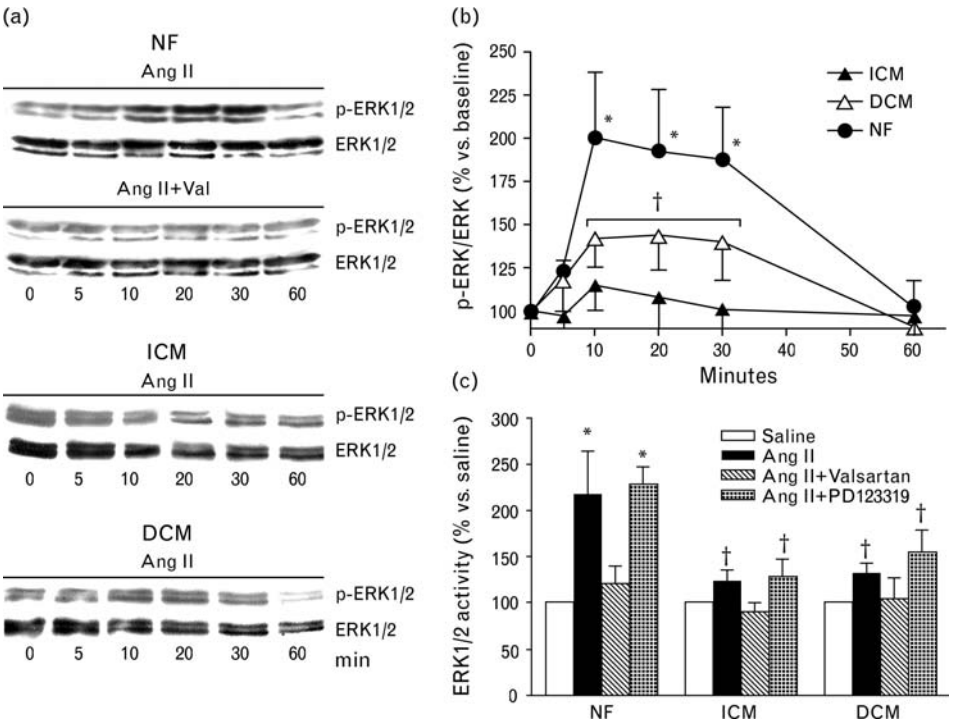
The release of big ET-1 induced by Ang II (100 nmol/l) in nonfailing myocytes was abolished by the ERK inhibitor PD98059 and reduced (50%) following the JNK inhibition (SP600125) (Fig. 1b). ERK inhibition also blocked the formation of IGF-I in Ang II-stimulated nonfailing myocytes (Fig. 1c). The effects of ERK antagonism on IGF-I and ET-1 expression in control myocytes were also confirmed at transcription level (Fig. 1a). Conversely, big ET-1 and IGF-I release were unaffected by p38 MAPK and JAK2 inhibitors (SB 203580 and AG 490, respectively) (Fig. 1b and c).

Ang II stimulation failed to enhance ET-1 and IGF-I production in human ventricular failing myocytes both from DCM and ICM hearts (Fig. 1) as previously demonstrated [5]. Reverse transcriptase-PCR studies confirmed that the altered response was not related to AT1 and AT2 changes in gene expression in isolated failing myocytes (Fig. 1d).

Altered angiotensin II-mediated extracellular signal-regulated kinase activation in human failing myocytes

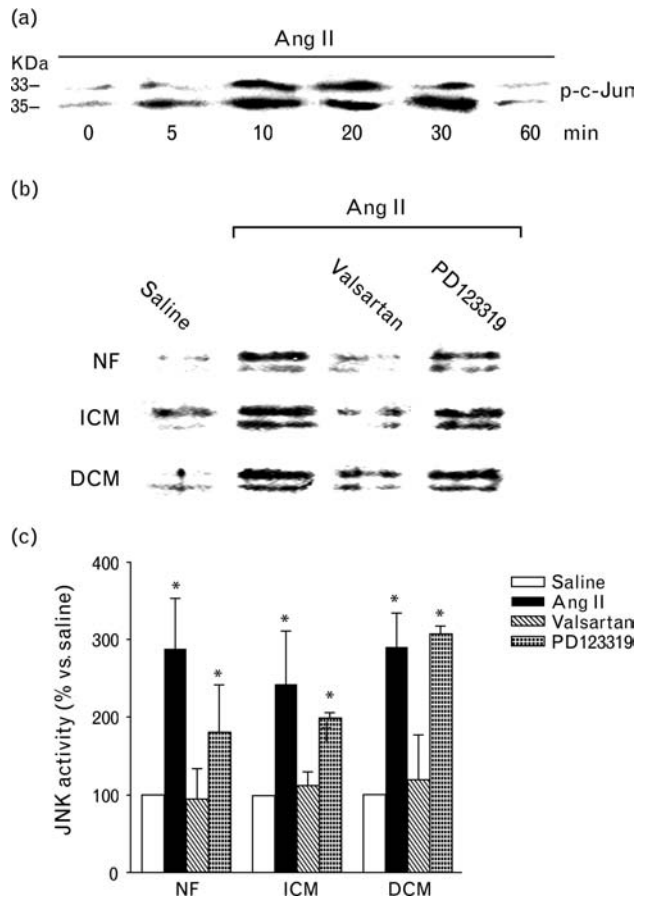
Ang II induced a prompt phosphorylation of ERK in nonfailing human ventricular myocytes, which significantly increased after 5 min (+24% vs. nonstimulated myocytes, $P < 0.05$), peaked at 10 min (+100%, $P < 0.05$) and returned to baseline at 60 min (Fig. 2a and b). ERK enzymatic activity was also enhanced by Ang II (+117% vs. controls after 10 min, $P < 0.05$) (Fig. 2c). The rises in both ERK phosphorylation and enzymatic activity were abolished by AT1 antagonism (Fig. 2a and c), whereas AT2 inhibition did not affect the ERK response to Ang II

Fig. 2



(a) ERK activation in Ang II (100 nmol/l) stimulated nonfailing (NF) myocytes. Representative western blots of phosphorylated (p-ERK) and total ERK in NF myocytes and in myocytes isolated from ICM and DCM patients are shown. ERK activation in NF is abolished by AT1 antagonism. (b) Time course of the ratios of phosphorylated to total ERK (p-ERK/ERK) for nonfailing (NF, $n = 6$) and failing myocytes (ICM, $n = 14$; DCM, $n = 19$). (c) Effects of AT1 (Valsartan) and AT2 (PD123319) selective antagonists on ERK1/2 activity in Ang II-stimulated myocytes in nonfailing (NF, $n = 6$) and failing myocytes (ICM, $n = 14$; DCM, $n = 19$). Ang II, angiotensin II; DCM, dilated cardiomyopathy; ERK, extracellular signal-regulated kinase; ICM, ischemic cardiomyopathy; NF, nonfailing; p-ERK, phosphorylated extracellular signal-regulated kinase. $^{*}P < 0.05$ vs. unstimulated myocytes; $^{\dagger}P < 0.05$ vs. NF myocytes.

Fig. 3



JNK activity in Ang II-stimulated (100 nmol/l) myocytes. (a) Time course of JNK activity assayed by phosphorylation of c-Jun. Representative western blot from nonfailing (NF) myocytes is shown. (b) Representative western blots showing the effects of AT1 (valsartan) and AT2 (PD123319) selective antagonists on nonfailing and failing (ICM and DCM) myocytes. (c) Bar graph shows the effects of AT1 (valsartan) and AT2 (PD123319) selective antagonists on JNK activity in Ang II-stimulated nonfailing (NF, $n=6$) and failing myocytes (ICM, $n=14$; DCM, $n=19$). Ang II, angiotensin II; DCM, dilated cardiomyopathy; ICM, ischemic cardiomyopathy; JNK, c-Jun N-terminal kinase; NF, nonfailing. $^*P<0.05$ vs. unstimulated myocytes.

(Fig. 2c). Conversely, Ang II stimulation failed to increase ERK phosphorylation at all experimental times in the failing myocytes from both DCM and ICM hearts (Fig. 2a and b). The ERK enzymatic activity of the failing myocytes only marginally increased in response to Ang II stimulation (+31% after 10 min, $P<0.05$) (Fig. 2c).

JNK activity in nonfailing myocytes was increased by Ang II stimulation in as little as 10 min (+160% vs. baseline, $P<0.05$); it peaked at 20 min (175%, $P<0.05$) and returned to baseline after 60 min (Fig. 3a). The enhancement in JNK activity was abolished by the AT1 antagonist (Fig. 3b and c). In contrast with the ERK pattern, JNK activity increased following Ang II stimulation in both DCM (+189%, $P<0.05$) and ICM myocytes (+142%, $P<0.05$) (Fig. 3b and c).

Ang II stimulation resulted in increased myocyte levels of MKP1 (60 min), MKP2 (60 min), and MKP3 (30–60 min) only in nonfailing myocytes (Fig. 4). Western blot analysis revealed higher levels of MKP1, MKP2, and MKP3 in failing myocytes than in nonfailing unstimulated ones (Fig. 4).

Discussion

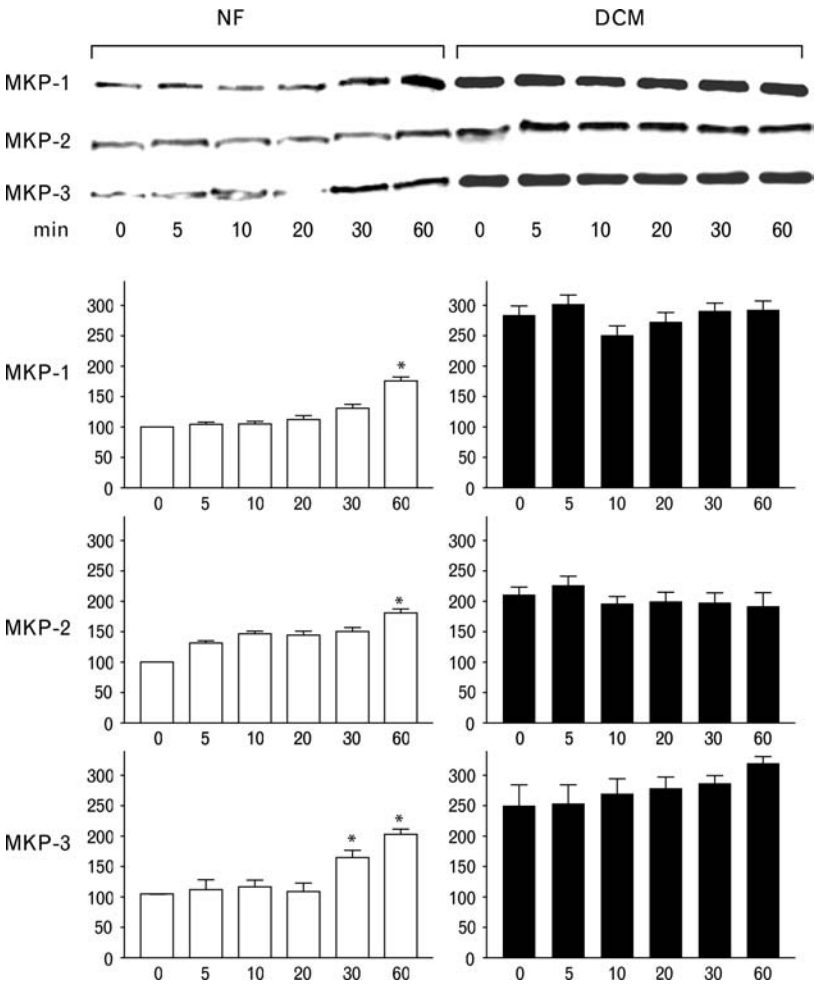
The present study reveals for the first time an altered myocyte intracellular response to Ang II in heart failure independent from changes in membrane receptors. The impaired Ang II-induced IGF-I and ET-1 release by human failing ventricular myocytes is associated with an impaired Ang II–ERK signaling.

Angiotensin II–extracellular signal-regulated kinase signaling mediates insulin-like growth factor-I and endothelin-1 release

Differently from what has been observed in other species, Ang II does not acutely enhance contractility of human and rat left ventricular myocytes [22–25]. In addition, Ang II 10–11 to 10–7 mol/l failed to elicit Ca^{2+} influx responses in adult rat ventricular cardiomyocytes [26]. However, when incubation time was prolonged (>15 min), Ang II induced a concentration-dependent increase in rat myocyte contractility, an effect completely blocked by ET-1 antagonists [15,27]. Likewise, the progressive increases in force and in Ca^{+} transients occurring over 15 min in stretched papillary muscles were blocked by both AT1 and endothelin receptor antagonists [28]. In-vivo studies in pig heart showed that the early release of Ang II stored in the cardiomyocyte cytoplasm granules [3] was followed by an increase in the mRNA of pre-proET-1 and angiotensinogen with final ET-1 release and recovery of cardiac contractility [2]. ET-1 gene expression and peptide release, as well as the recovery of contractility, were all abrogated by AT1 receptor blockade [3]. Therefore, the participation of growth factors in mediating the inotropic effect of Ang II was demonstrated in ventricular myocytes, multicellular preparations and in in-vivo studies. It is now considered that Ang II-induced release of ET-1 activates the Na^{+}/H^{+} exchanger in an autocrine/paracrine fashion promoting the influx of Ca^{2+} [27] and intracellular alkalinization, which further improves the responsiveness of myofilaments to calcium [29]. These changes finally result in increased contractility [30]. However, the relative role of the different intracellular pathways activated by Ang II in causing ET-1 release by human myocytes remains to be defined.

According to the present findings, ERK antagonism completely abolished ET-1 and IGF-I peptide release, extending previous observations obtained in neonatal rat myocytes to adult human cells [31]. Conversely, JNK inhibition caused only a minor reduction in ET-1 release with no effects on IGF-I release. In addition, the lack of increase in

Fig. 4



Effects of Ang II (100 nmol/l) stimulation on the expression of MKP1, MKP2, and MKP3 in myocytes isolated from nonfailing (NF, left) and DCM (right) hearts. Upper panels: representative western blots. Lower graphs: mean levels of MKP in NF (empty bars) and DCM myocytes (filled bars) at different times. Values are expressed as percentage vs. baseline values in unstimulated cells. DCM, dilated cardiomyopathy; MKP, mitogen-activated protein kinase phosphatases. $*P < 0.05$ vs. baseline values.

ET-1 and IGF release following JNK inhibition excludes a possible inhibitory effect of JNK on growth factor release. In conclusion, these experiments confirm the redundant role of JNK in ET-1 release [32,33] that is conceivable with the effect of JNK downstream from ERK [34].

Altered angiotensin II–extracellular signal-regulated kinase activation in failing myocytes

Evidence obtained in humans suggests that this mechanism may be altered in the progression to failure. The reduction in cardiac contractility characterizing the transition from hypertrophy to failure in patients with aortic valve disease was indeed found to be associated with reduced cardiac production of IGF-I and ET-1 [4], notwithstanding the progressive increase in Ang II cardiac generation. In patients with heart failure, the progressive reduction in contractility, marked by the increase in left ventricular stress, was finally associated with reduced cardiac production of growth factors (IGF-I and ET-1) and progressive increase in Ang II cardiac generation [5]. In failing hearts, a reduced myocyte expression of ET-1 and IGF-I genes was observed by in-situ hybridization, and the capability of Ang II to induce ET-1 and IGF-I gene activation and peptide release was finally lost in ventricular myocytes isolated from end-stage failing hearts [5].

The present results reveal, for the first time, that this pattern of response is associated with a blunted ERK activation and a preserved JNK response to Ang II stimulation. No significant differences were found between DCM and ICM myocytes, thus indicating that ERK impairment is inherent in heart failure.

ERK is now considered to be a protective signaling pathway [9,10], its deletion predisposing the heart to

decompensation and failure [17]. In addition, IGF-I [13] and ET-1 [14] regulate myocyte survival, so that the altered myocyte response to Ang II might favor the progression to failure. In our nonfailing myocytes, the MKP activation occurring at 30–60 min closely corresponds to ERK and JNK dephosphorylation. MKP1, MKP2, and MKP3 have been found to be increased in human failing hearts [7] and according to the present data are also increased in failing myocytes. The relative excess of phosphatases might thus contribute to ERK impairment in human failing myocytes. However, the lack of differences between the pattern of the three MKP investigated, as well as the normalcy of Ang II–JNK activation in failing myocytes, does not support a primary role for the altered MKP pattern in determining the ERK impairment.

The observed reduced ERK response to Ang II might also attract interest in possible alterations in Ang II-induced Ca mobilization in failing myocytes, an aspect indeed not covered by our experiments. Unlike JNK, the ERK1/2 response to Ang II was indeed reported to be influenced by the release of calcium from inositol 1,4,5-trisphosphate (IP3)-sensitive intracellular stores (sarcoplasmic reticulum) both in vascular smooth muscle cells [35,36] and in cardiomyocytes isolated from neonatal rats [37]. However, although a reduced calcium release from intracellular stores was consistently observed in human failing ventricular myocytes [38–41], we did not specifically investigate Ca mobilization in our study, so the possible participation of this mechanism can only be hypothesized. Therefore, these experiments, although not clarifying in detail the mechanisms involved in the reduced ERK response in failing myocytes, provide evidence in support of a direct link between the impairment of an important intracellular pathway and reduced growth factor formation in human failing myocytes.

The selective ERK impairment we observed in failing cardiomyocytes differs from studies obtained in homogenated human failing hearts, the latter giving conflicting results. Samples obtained from patients with heart failure either due to coronary artery disease or idiopathic dilated cardiomyopathy revealed an increase in all three MAPKs [42]. In comparison, levels of activated ERK were unchanged in heart samples from patients with heart failure secondary to ischemic heart disease, whereas levels of JNK and p38 MAPK activation were significantly increased [6]. In patients with idiopathic dilated cardiomyopathy, no increase in ERK activity in failing hearts was reported [7], notwithstanding a three-fold increase in the protein level for ERK. In the same study, JNK and p38 MAPK protein levels were not different in failing hearts although the activity of both was decreased [7]. More recently, reduced ERK activation was found in the hearts of hypertensive patients who developed heart failure [43]. Different patterns of MAPK activation in homogenated heart were thus observed in patients with different causes of the disease. Indeed, no true discrepancy may exist because we investigated the pattern of response to Ang II rather than the 'status' of the whole myocardium and the two aspects cannot be compared. The use of isolated human ventricular myocytes certainly constitutes an advance because the present findings suggest that, regardless of the cause of the disease, the impaired Ang II–ERK signaling might play a causative role in the loss of Ang II physiological activity on human failing myocytes.

Rather than indicating a total downregulation of Ang II effects, the present findings support the existence of a complex alteration in Ang II-mediated intracellular signaling in failing cardiomyocytes. In other cell types, the signaling cascade linking the activation of the nuclear transcriptional changes and cell growth to Ang II type I receptor stimulation was found to include the phosphorylation of JAK2, a soluble tyrosine kinase [44] also involved in the transmission of the inflammatory processes [45]. Recent data showed that Ang II was unable to activate JAK2 in human nonfailing myocytes, whereas it did activate the same pathway in the failing myocytes [8]. The enhanced ROS generation appeared to be the essential factor for JAK2 activation in the failing myocytes [8]. Although Ang II-mediated ROS generation was also found to be involved in JNK activation with no effect on ERK1/2 [46], we did not extensively investigate the role of ROS in the preserved JNK response due to the limited availability of human myocytes. In conclusion, the complex of these data clearly indicates the occurrence of a derangement of Ang II signaling in failing cardiomyocytes, supporting the use of Ang II antagonism in this clinical setting.

Study limitations

The possibility that we isolated a selected group of myocytes cannot be excluded although a similar unintentional selection has to be hypothesized also in controls. However, the selected population, although 'robust' enough to survive the stress of isolation, shows different characteristics when compared with adult nonfailing myocytes. Therefore, although present results cannot be extended to the organ level, they identify important functional aspect of active cardiac cells, that is, the capability of myocytes to react to the enhanced Ang II production in heart failure [5].

Ang II-mediated signal transduction to AT1 receptors may result from the effects of Ang II on contaminating fibroblasts. A high prevalence of contaminating fibroblasts (>10%) has been reported when neonatal rat cardiac cells are isolated and cultured [47]. Although the possibility that myocyte samples may be contaminated by other cell types cannot be excluded in any study investigating isolated myocytes, our studies were performed with the use of adult cells, and our cell suspensions were preplated to reduce the number of

contaminating fibroblasts because, unlike myocytes isolated from neonatal rats, adult myocytes do not attach to culture plates. In addition, we used freshly isolated cells to avoid the progressive increase in the prevalence of contaminating fibroblasts with culture [48]. Finally, cell suspensions obtained in the three groups did not significantly differ with regard to the number of nonmyocyte cells identified by their appearance under phase contrast microscopy and by immunocytochemical staining. Failing myocytes were obtained from patients receiving a variety of drugs, including AT1 antagonists. A possible interference of retained AT1 antagonists with Ang II–ERK activation can be excluded because the Ang II–JNK signaling was unchanged in the same human failing myocytes.

Conclusion

The present findings reveal, for the first time, an important derangement of Ang II signaling in human failing ventricular cardiomyocytes with a severe impairment of the ERK pathway. Although the present cross-sectional study does not allow the role of ERK impairment in the single patient to be defined clearly, the altered myocyte response might favor the progression to failure.

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