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PAPER

Impaired JAK2-induced activation of STAT3 in failing human myocytes

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Although angiotensin (Ang)II-induced Janus-activated kinase (JAK)2 phosphorylation was reported to be enhanced in failing human cardiomyocytes, the downstream balance between cardio-protective (signal transducer and activator of transcription-STAT3) and the pro-inflammatory (STAT2 and STAT5) response remains unexplored. Therefore STATs phosphorylation and putative genes overexpression following JAK2 activation were investigated in isolated cardiomyocytes obtained from failing human hearts ($n = 16$), and from non-failing(NF) hearts of humans (putative donors, $n = 6$) or adult rats. In NF myocytes Ang II-induced JAK2 activation was followed by STAT3 phosphorylation (186 ± 45% at 30 min), with no STAT2 or STAT5 response. The associated B cell lymphoma (Bcl)-xL overexpression (1.05 ± 0.39 fold) was abolished by both JAK2 and extracellular signal-regulated kinase (ERK)1/2 inhibitors (AG490, 10 μM, and PD98059, 30 μM, respectively), whereas Fas ligand (Fas-L) response (0.91 ± 0.21 fold) was inhibited only by p38MAPK antagonism (SB203580, 10 μM). In failing myocytes Ang II-induced JAK2 activation was followed by STAT2 (237 ± 38%) and STAT5 (222 ± 31%) phosphorylation, with no STAT3 response. No changes in Bcl-xL expression were observed, and the associated Fas-L gene overexpression (1.14 ± 0.27 fold) being abolished by p38 mitogen-activated protein kinase (MAPK) antagonism. The altered JAK2 induced STATs response in human failing cardiomyocytes may be of relevance for the progression of cardiac dysfunction in heart failure.

Introduction

Recent evidence shows that the activation of the signal transducer and activator of transcription (STAT)3 in response to various pathophysiologic stimuli promotes cardiomyocytes survival and hypertrophy as well as cardiac angiogenesis, suggesting that STAT3 is beneficial to the heart.[1] In human heart failure the expression and phosphorylation levels of STAT3 were reported to be reduced.[2] Mice with postnatal cardiomyocyte-specific disruption of STAT3 develop dilated cardiomyopathy and heart failure, which leads to premature death,[3,4] so that the possibility that the decreased activation of STAT3 may contribute to the development of heart failure in patients was raised.

STAT3 activation is mediated by Janus-activated kinase (JAK)2 through phosphorylation of receptor tyrosine residues.[5,6] In addition to tyrosine phosphorylation STAT3 was also reported to require extracellular signal-regulated kinase (ERK)1/2 induced serine phosphorylation to exert full transcriptional activity.[7] JAK2 response to Angiotensin (Ang)II was found to be enhanced in human failing cardiomyocytes,[8] where conversely ERK1/2 response to Ang II was reported to be impaired.[9] However, information regarding events downstream JAK2 in human failing cardiomyocytes, and in particular the STATs response to Ang II, is lacking. The signaling pathway activated by JAK2 may indeed lead to different final responses depending on the selective phosphorylation of different elements of the STAT family. Besides the beneficial effect played by STAT3, other STATs have been reported to play a role in maladaptive signaling, such as STAT2, which is being involved in type I interferon signaling,[10,11] and STAT5 reported to stimulate angiotensinogen promoter activity in models of ischemia/reperfusion.[12]

The present study has therefore been designed to investigate in Ang II stimulated cardiomyocytes (1) the relationship between the activation of STAT2, STAT3, or STAT5 and the expression of interferon regulatory factor-1 (IRF-1),[13]

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Fas ligand (Fas-L)[14] and B-cell lymphoma-xL (Bcl-xL);[15] (2) the crosslink between JAK/STAT and mitogen-activated protein kinase (MAPK) (ERK1/2 and p38MAPK) in the control of gene expression; and finally (3) changes in the downstream balance between cardio-protective STAT3, and the pro-inflammatory STAT2 and STAT5 pathway in heart failure.

Experimental

Source of tissues

Human hearts were obtained from patients who underwent cardiac transplantation due to dilated (DCM, $n = 8$) or ischemic cardiomyopathy (ICM, $n = 8$), and from 6 putative organ donors with no history or signs of heart disease (NF = non-failing hearts) (Table 1). Subjects with arterial hypertension, or with a recent history of myocardial infarction (less than 6 months ago), or echocardiographic evidence of valve or congenital heart disease were not considered for the study. Diagnosis of ICM or DCM was based on clinical and echocardiographic examination, cardiac catheterization, and coronary angiography. Myocardial tissue was obtained from a portion of the left ventricular free wall during cardiac transplantation. The protocol of this study complies with the principles of the Helsinki declaration and was approved by our institution. All patients gave their informed written consent to participate and to have their hearts used for the study.

Non-failing ventricular myocytes were also obtained from the hearts of male adult Wistar Han™ rats (275 to 300 g body weight) (Harlan Laboratories, Milan, Italy). Our investigations conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996), and all procedures were performed in accordance with the animal care guidelines of Federation of Laboratory Animal Science Associations.

Myocytes isolation

All studies were performed on freshly-isolated ventricular myocytes. After explantation, human hearts were placed in cardioplegic solution and immediately transferred to the laboratory where myocytes were immediately isolated by an enzymatic digestion method as previously described.[8,16] Briefly, to clear the myocardial vasculature of retained blood cells, a coronary artery branch was cannulated and tissue was perfused for 10–15 min with a calcium-free basic buffer (Basic Buffer, BB), composed of Jocklik modified minimal essential medium (MEM Joklik; Sigma M0518, Sigma Chemical Co., St. Louis, Missouri, USA) supplemented with glutamine (Sigma G5763; 0.3 g L^{-1}), taurine (Sigma T0625; 1.25 g L^{-1}), HEPES (Sigma H3375; 2.9 mM), insulin (20 U L^{-1}), 5 mL L^{-1} Penicillin-Streptomycin (Sigma P3539; 50 U mL^{-1} and 0.05 mg mL^{-1}, respectively), and $CaCl_2$ (7.5 μM), pH 7.4. The basic buffer has previously been filtered through filters of 0.2 μm pore size. Perfusion was then switched to collagenase solution, composed of 0.5 mg mL^{-1} of Worthington type II collagenase (100 U mL^{-1}) in BB supplemented with $CaCl_2$ 30 μM (20 mL min^{-1} for 20–25 min). The collagenase-perfused tissue was then minced and the tissue collected in tubes containing BB supplemented with bovine serum albumin (0.5%), $CaCl_2$ (0.3 mM), and taurine (10 mM). Individual myocytes were then released from the tissue by mechanical agitation. The suspension was filtered through 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.

Rats were injected intraperitoneally with 0.5 mL heparin (Epsoclar 5000 U mL^{-1}) 30 min prior to killing by decapitation. Then aorta was cannulated and perfused with a calcium-free buffer (blood washout) for 10–15 min and then with Worthington type II collagenase (100 U mL^{-1}) for 15 min. The collagenase-perfused tissue was then minced and shaken in a resuspension buffer (HEPES-MEM buffer supplemented with bovine serum albumin 0.5%, 0.3 mM $CaCl_2$, and 10 mM taurine).

Typical preparations contained 60–70% rod-shaped, quiescent, calcium-tolerant and trypan blue-excluding myocytes which had well-defined, regular cross-striations and sarcomere patterns (Fig. 1). According to their appearance under phase contrast microscopy and with immunocytochemical staining (antisarcomeric alpha-actinin and antiskeletal myosin antibodies; both from Sigma Chemical Co.), non-myocyte cells were found to account for less than 2% of the total cells.[8,16]

Table 1 Characteristics of subjects investigated

Clinical characteristics	NF	ICM	DCM	ANOVA $P<$
Age, years	46 ± 7	55 ± 7	55 ± 9	ns
Gender (M/F)	5/1	5/3	6/2	ns
BSA, m^2	1.84 ± 0.12	1.90 ± 0.15	1.80 ± 0.07	ns
SAP, mmHg	121.7 ± 14.2	115.7 ± 3.4*	113.8 ± 6.1*	0.05
LVEDDI, mm m^{-2}	28.7 ± 2.5	45.1 ± 5.2*	48.7 ± 5.0*	0.05
LVESDI, mm m^{-2}	19.0 ± 3.1	31.9 ± 3.6*	36.6 ± 6.4*	0.05
EF (%)	62.7 ± 4.5	28.2 ± 4.3*	21.7 ± 4.5*	0.05
MPAP, mmHg	19.3 ± 1.7	34.7 ± 6.7*	34.8 ± 5.2*	0.05
CI, L m^{-2} min^{-1}	2.86 ± 0.13	1.81 ± 0.50*	1.87 ± 0.23*	0.05

NF, non-failing; ICM, ischemic cardiomyopathy; DCM, dilated cardiomyopathy; BSA, body surface area; SAP, systolic aortic pressure; LVEDDI, left ventricular end-diastolic diameter index; LVESDI, left ventricular end-systolic diameter index; EF, ejection fraction; MPAP, mean pulmonary arterial pressure; CI, cardiac index. Data are expressed as means ± SD or number of patients. * = $P < 0.05$ *vs.* NF subjects, at the multiple comparison test (Tukey).

Myocytes stimulation

After isolation, myocytes (10^5 cell mL^{-1}) were suspended in Dulbecco's Modified Eagle' Medium (DMEM) (pH 7.2–7.4)

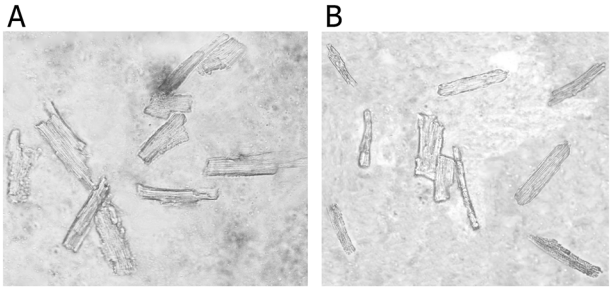

Fig. 1 Representative human myocytes enzymatically isolated from two left ventricles of non-failing (A, tentative donor) and failing (B, dilated cardiomyopathy) human hearts.

supplemented with 100 U mL^{-1} penicillin G and 0.1 mg mL^{-1} streptomycin and incubated under an atmosphere of 95% air plus 5% CO_2 at 37 °C. After 30 min, the medium was changed with DMEM containing normal (NG, 5 mM) or high glucose (HG, 25 mM) concentrations corresponding to plasma levels of 100 and 450 mg dL^{-1}, respectively (2 h pre-incubation under an atmosphere of 95% air plus 5% CO_2 at 37 °C).[8]

Myocytes were then stimulated with Ang II (100 nM)[8,16] or interleukin-6 (IL-6, 10 ng mL^{-1}). To investigate the relative role of the two angiotensin receptor subtypes, myocytes were preincubated for 30 min with selective Ang II type 1 (AT1) or Ang II type 2 (AT2) receptor antagonists (Valsartan, 1 μM or PD123319, 1 μM, respectively). In addition, we performed experiments in the presence of a flavoprotein inhibitor (diphenyleneiodonium [DPI], 100 μM), and an inhibitor of mitochondrial reactive oxygen species (ROS) production (rotenone, 5 μM). To investigate the effects of ERK1/2, JAK2, and p38MAPK inhibition, myocytes were preincubated for 60 min with ERK1/2 inhibitor (PD98059, 30 μM), JAK2 inhibitor (AG490, 10 μM), or p38MAPK inhibitor (SB203580, 10 μM).

At baseline and after 15, 30 and 60 min, the conditioned media and myocytes were separated by centrifugation and immediately frozen at –80 °C.

Western blot analysis

Myocytes suspension was homogenized in RIPA buffer containing protease inhibitor cocktail (Sigma-Aldrich, Milan, Italy) and phosphatase inhibitor cocktail (Sigma-Aldrich).[8] Solubilized myocytes proteins (25 μg proteins) were separated with 8% SDS-PAGE and transferred to a nitrocellulose membrane (Hybond-ECL, Amersham Biosciences, Piscataway, New Jersey, USA). The membrane was blocked with 5% fat-free milk followed by incubation with 1 : 1000 dilution of polyclonal rabbit anti-JAK2 (Santa Cruz Biotechnology, Inc., CA, USA), polyclonal rabbit anti-phospho-JAK2 (Tyr1007/1008) (Upstate Biotechnology, Lake Placid, NY, USA), polyclonal rabbit anti-STAT2 (Santa Cruz Biotechnology), polyclonal rabbit anti-phospho-STAT2 (Tyr690) (Cell Signaling Technology, Danvers, MA, USA), polyclonal rabbit anti-STAT3, polyclonal rabbit anti-phospho-STAT3 (Tyr705) (Cell Signaling Technology), polyclonal rabbit anti-STAT5, or polyclonal rabbit anti-phospho-STAT5 IgG (Tyr694) (Cell Signaling Technology).

After washing, the membrane was blocked with the corresponding secondary HRP-conjugated IgG and the specific bound antibody was visualized using a chemioluminescent detection system (ECL detection reagents, GE Healthcare, formerly Amersham Biosciences, Milan, Italy). The amount of each band was quantified using a densitometer software (Quantity One, Bio-Rad Laboratories, Milan, Italy). The membranes previously incubated with primary antibodies for phosphorylated proteins were incubated in stripping buffer and then reprobed with the corresponding antibodies against the native proteins. The intensity of the phospho-immunostained bands were normalized with the total protein intensities measured by non-phospho-immunostained bands from the same blot in order to normalize to the non-phosphorylated protein. For each protein results are then expressed as the phosphorylated to total ratio.

Gene expression (reverse transcription-PCR)

The total amount of RNA was extracted with a NucleoSpin RNA II Kit (Macherey-Nagel, Düren, Germany) and then reverse transcribed using a TaqMAN Reverse Transcription

Table 2 Sequences (S, sense; AS, antisense), cDNA sizes and thermal profiles of primers used for investigation of Fas-L, Bcl-xL, IRF-1 and GAPDH gene expression in human and rat cardiomyocytes

Primers	Sequence 5′-3′	Size/bp	Hot start/ °C s^{-1}	Thermal profiles/°C s^{-1}			Final extension/ °C min^{-1}	No. of cycles
				Denaturation	Annealing	Extension		
Human Fas-L								
S	AGGCAAGTCCAACTCAAGGTCC	238	95/300	95/30	58/30	72/60	72/7	35
AS	CATCTTCCCCTCCATCATCACC							
Bcl-xL								
S	GGAGCTGGTGGTTGACTTTCT	379	94/120	94/30	52/60	72/120	72/7	40
AS	CCGGAAGAGTTCATTCACTAC							
IRF-1								
S	CTTAAGAACCAGGCAACCTCTGCCTTC	406	95/60	95/60	55/60	72/60	72/7	25
AS	GATATCTGGCAGGGAGTTCATG							
GAPDH								
S	CATGGCACCGTCAAGG	245	95/300	95/30	55/30	72/30	72/7	25
AS	CACCATGGGGGCATCAGC							
Rat Fas-L								
S	GGAATGGGAAGACACATATGGAACTGC	238	94/180	94/300	60/60	72/60	72/7	35
AS	CATATCTGGCCAGTAGTGCAGTAATTC							
Bcl-xL								
S	AGGATACAGCTGGAGTCAG	417	95/180	95/50	61/50	72/50	72/7	31
AS	TCTCCTTGTCTACGCTTTCC							
IRF-1								
S	CTTCAGAGCTTAGGAGGCAGGGTCT	450	94/180	94/30	62/60	72/120	72/7	28
AS	AGCAGGCACAGGGCAAGGCACTATA							
GAPDH								
S	GGTCGGTGTGAACGGATTTG	350	94/300	94/60	60/60	72/60	72/10	30
AS	GTGAGCCCCAGCCTTCTCCAT							

Reagents kit (Applied Biosystem, Monza, Italy) according to the manufacturer's protocol. cDNA was then amplified using specific primer sequences, thermal profiles and number of cycles for each investigated gene (Fas-L, Bcl-xL, IRF-1, and GAPDH) (Table 2). Expression of these mRNAs was examined using ethidium bromide-stained agarose gel electrophoresis. The band intensity of the PCR products was quantified by using the public domain NIH Image program (ImageJ).

Statistical analysis

Statistical analysis was performed using SPSS Statistical Software 14.0 (SPSS Inc., Chicago, IL). Data are expressed as mean ± SD. Comparisons between groups were performed using one-way analysis of variance, followed by the Tukey multiple-range comparison test, as appropriate. In addition, the response to different stimulations, expressed as a ratio to one point (such as the signal measured in the presence of buffer only), were compared with a non-parametric test (Kruskal–Wallis). A value of $P < 0.05$ was considered statistically significant.

Results and discussion

Ang II-induced STAT3 phosphorylation and gene expression in non-failing myocytes

As expected, Ang II-induced JAK2 activation (187 ± 40% and 213 ± 43% *vs.* buffer at 30 and 60 min, respectively; $P < 0.05$ for both at the Tukey *post hoc* test) was closely associated with STAT3 Tyr705 phosphorylation only in the presence of HG concentration (186 ± 45% and 206 ± 47% *vs.* buffer at 30 and 60 min, respectively; $P < 0.05$ for both) (Fig. 2A). Differently from Ang II, IL-6-induced STAT3 phosphorylation occurred independently by glucose concentrations (positive control, Fig. 2B). STAT3 phosphorylation was selectively inhibited by AT_1 receptor antagonist, JAK2 inhibitor, and by inhibitors of ROS generation (DPI; Rotenone), being not affected by ERK1/2 inhibition (Fig. 2B). No STAT2 and STAT5 activation was observed in non-failing human cardiomyocytes under any experimental conditions (Fig. 2B).

Experiments performed in rat myocytes showed that Ang II induced the expression of Fas-L, independently of glucose

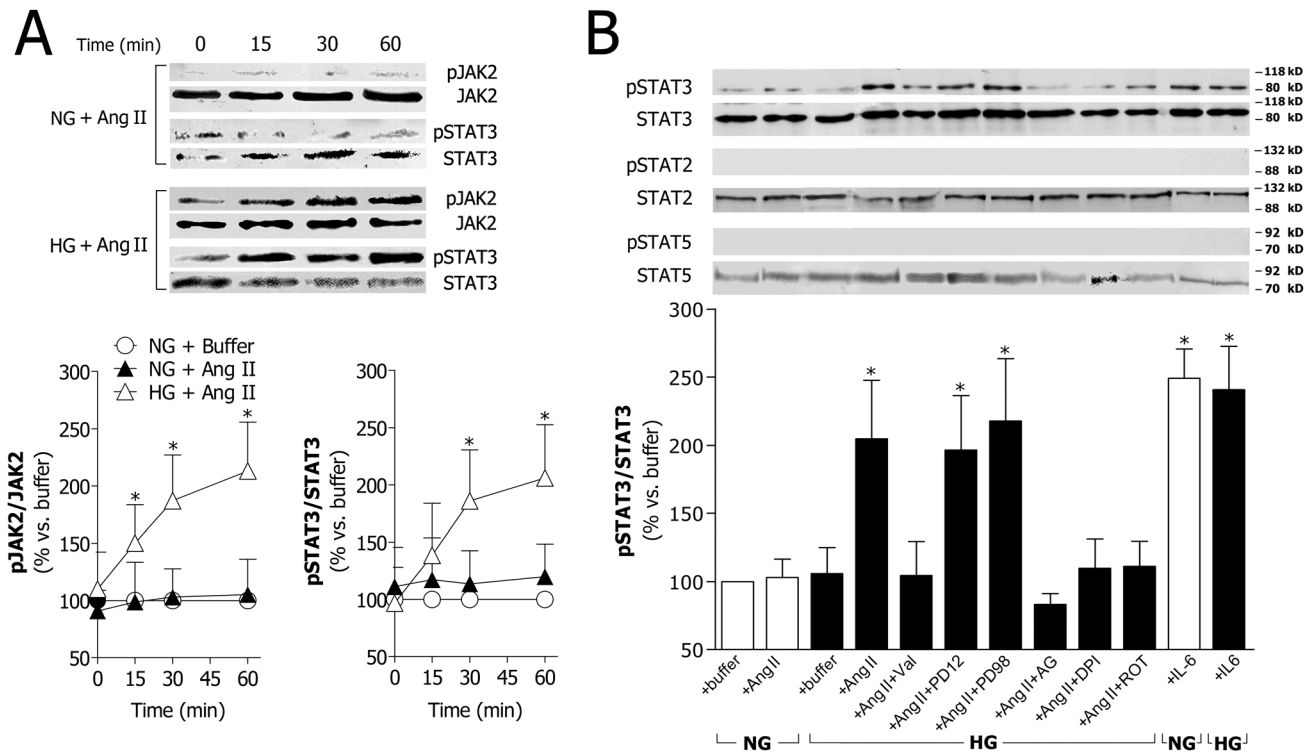

Fig. 2 In non-failing human cardiomyocytes Ang II-induced STAT3 tyrosine phosphorylation in the presence of high glucose concentrations is mediated by AT1 receptor subtypes *via* JAK2 activation and ROS generation. (A) Upper panels: representative Western blots performed by using anti-phospho-JAK2 (pTyr1007/1008 JAK2), anti-JAK2 (JAK2), anti-phospho-STAT3 (pTyr705 STAT3), and anti-STAT3 (STAT3) antibodies on lysates of myocytes at baseline and after 15, 30, and 60 min of stimulation with Ang II (100 nM) in the presence of normal (NG, 5 mM), or high glucose (HG, 25 mM) concentrations (2 h of pre-incubation with glucose). Lower graphs: time course of changes of phosphorylated-to-total JAK2 (pTyr1007/1008 JAK2/JAK2, left) and STAT3 (pTyr705 STAT3/STAT3, right) ratios induced by buffer or by Ang II in the presence of NG or HG. Data, expressed as per cent increase relative to the buffer, are means ± SD of six separate experiments (* = $P < 0.05$ *vs.* buffer at Tukey *t* test). (B) Upper panels: representative Western blots performed by using anti-phospho-STAT3 (pTyr705 STAT3), anti-STAT3 (STAT3), anti-phospho-STAT2 (pTyr690 STAT2), anti-STAT2 (STAT2), anti-phospho-STAT5 (pTyr694 STAT5), anti-STAT5 (STAT5) antibodies on lysates of myocytes preincubated with NG or HG buffer. Effects of inhibition of AT1 (Val,Valsartan, 1 μM) or AT2 receptor subtypes (PD12, PD123319, 1 μM), ERK1/2 (PD98, PD98059, 30 μM), JAK2 (AG, AG490, 10 μM) or ROS generation (DPI, 100 μM; ROT, Rotenone, 5 μM) added 30 or 60 min before Ang II stimulation in HG buffer. Effects of interleukin-6 (IL-6, 10 ng mL^{-1}; 30 min) in the presence of NG or HG buffer. Lower graphs: Phosphorylated-to-total STAT3 ratio (pTyr705 STAT3/STAT3) quantitated from all experiments. Data, expressed as per cent increase relative to the buffer, are means ± SD of six separate experiments (* = $P < 0.05$ *vs.* buffer at Kruskal–Wallis test).

concentration (0.86 ± 0.39-fold and 0.83 ± 0.35-fold *vs.* buffer at 30 and 60 min, respectively; $P < 0.05$ for both at the Tukey *post hoc* test), and the expression of Bcl-xL only in the presence of HG concentration (1.05 ± 0.39-fold and 0.90 ± 0.36-fold *vs.* buffer at 30 and 60 min, respectively; $P < 0.05$ for both) (Fig. 3A). No IRF-1 response was detectable under any experimental conditions (Fig. 3B). Fas-L gene response to Ang II was abolished by p38MAPK inhibition, whereas ERK1/2 or JAK2 inhibition had no effects (Fig. 3B). Conversely, Bcl-xL gene overexpression was almost completely inhibited by both JAK2 and ERK1/2 antagonism, with no effect being played by p38MAPK inhibition (Fig. 3B).

Ang II-induced STATs phosphorylation and gene expression in human heart failure

In failing myocytes, Ang II-induced JAK2 activation was followed by STAT2 Tyr690 phosphorylation, both in ICM (237 ± 38% and 248 ± 28% *vs.* buffer at 30 and 60 min, respectively; $P < 0.05$ for both at the Tukey *post hoc* test), and DCM cardiomyocytes (230 ± 51% and 228 ± 13% *vs.* buffer at 30 and 60 min, respectively; $P < 0.05$ for both) (Fig. 4A). Similar findings were shown for Ang II-induced STAT5 Tyr694 phosphorylation, both in ICM (222 ± 31% and 260 ± 25% *vs.* buffer at 30 and 60 min, respectively; $P < 0.05$ for both) and DCM cardiomyocytes (222 ± 36% and 254 ± 40% *vs.* buffer at 30 and 60 min, respectively; $P < 0.05$ for both) (Fig. 4A). No STAT3 activation was observed in failing human cardiomyocytes under any experimental conditions (Fig. 4A and B). Differently from what was observed in non-failing myocytes, Ang II-induced JAK2 tyrosine phosphorylation in failing human myocytes was independent of glucose concentration (Fig. 4B). Likewise, Ang II-induced STAT2 and STAT5 phosphorylation occurred independently of glucose concentration (Fig. 4B). The preincubation of failing

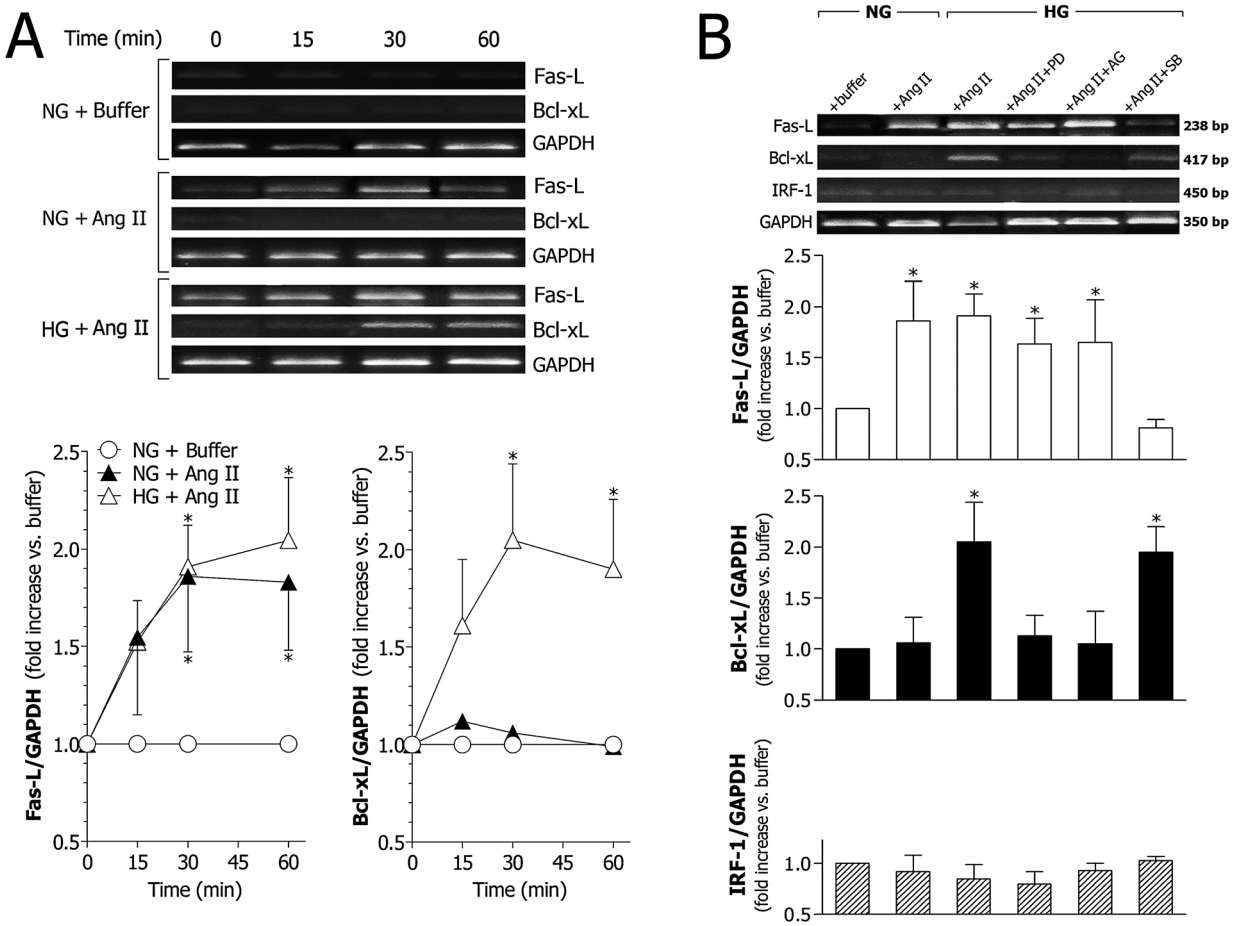

Fig. 3 In non-failing rat cardiomyocytes Ang II-induced Fas-L mRNA overexpression occurs independently of ERK1/2 and JAK2 activation, being inhibited by p38MAPK antagonism. Conversely, Bcl-xL gene overexpression is inhibited by both ERK1/2 and JAK2 antagonism. No response of IRF-1 gene was observed. (A) Upper panels: Representative RT-PCR experiments of Fas-L, Bcl-xL, and GAPDH mRNA expression in rat myocytes at baseline and after 15, 30, and 60 min of stimulation with buffer or Ang II (100 nM) in the presence of normal (NG, 5 mM), or high glucose (HG, 25 mM) concentrations (2 h of pre-incubation with glucose). Lower graphs: time course of genes (Fas-L and Bcl-xL)/GAPDH mRNA ratio expressed as fold increase relative to the buffer, set at 1.0. Data are means ± SD of six separate experiments (* = $P < 0.05$ *vs.* buffer at the Tukey *t* test). (B) Upper panels: representative RT-PCR experiments of Fas-L, Bcl-xL, IRF-1, and GAPDH mRNA expression 30 min after stimulation with buffer or Ang II in the presence of NG or HG buffer. Effects of inhibition of ERK1/2 (PD, PD98059, 30 μM), JAK2 (AG, AG490, 10 μM), and p38MAPK (SB, SB203580, 10 μM) added 60 min before Ang II stimulation in HG buffer. Lower graphs: fold increase of Fas-L, Bcl-xL, and IRF-1 mRNA expression (gene/GAPDH ratio) induced by Ang II (30 min) relative to the buffer, set at 1.0. Data are means ± SD of six separate experiments (* = $P < 0.05$ *vs.* buffer at the Kruskal–Wallis test).

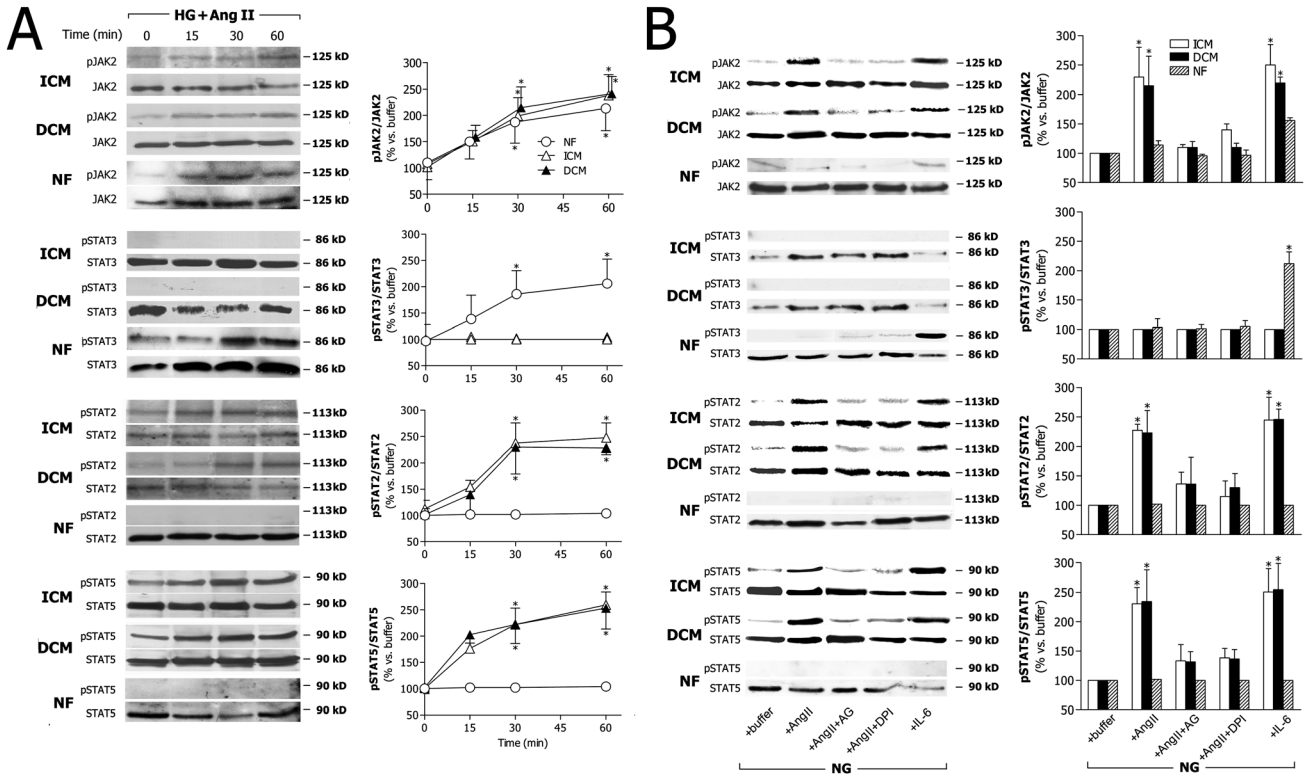

Fig. 4 Ang II-stimulation in human failing cardiomyocytes (obtained from patients with ischemic, ICM $n = 8$, or dilated, DCM $n = 8$, cardiomyopathy) is followed by STAT2 and STAT5 tyrosine phosphorylation whereas the STAT3 response observed in non-failing (NF) cardiomyocytes is abolished. (A) Left panels: representative Western blots performed by using anti-phospho-JAK2 (pTyr1007/1008 JAK2), anti-JAK2 (JAK2), anti-phospho-STAT3 (pTyr705 STAT3), anti-STAT3 (STAT3), anti-phospho-STAT2 (pTyr690 STAT2), anti-STAT2 (STAT2), anti-phospho-STAT5 (pTyr694 STAT5), anti-STAT5 (STAT5) antibodies on lysates of human NF and failing (ICM, DCM) myocytes stimulated with Ang II (100 nM) in the presence of high glucose (HG, 25 mM) concentrations (2 h of pre-incubation with glucose). Right graphs: time course of changes of phosphorylated-to-total JAK2 (pTyr1007/1008 JAK2/JAK2), STAT3 (pTyr705 STAT3/STAT3), STAT2 (pTyr690 STAT2/STAT2), STAT5 (pTyr694 STAT5/STAT5) ratios induced by Ang II in the presence of HG. Data are means ± SD of three separate experiments for NF cells and of eight separate experiments for both ICM and DCM cells (* = $P < 0.05$ *vs.* buffer at the Tukey *t* test). (B) Left panels: representative Western blots of the effects of JAK2 (AG, AG490, 10 μM) and ROS antagonism (DPI, 100 μM) on JAK2, STAT3, STAT2, and STAT5 phosphorylation in NF, ICM and DCM ventricular myocytes stimulated with Ang II (30 min) in the presence of normal glucose (NG, 5 mM) concentrations. Negative and positive controls are represented by stimulation with buffer and interleukin-6 (IL-6, 10 ng mL^{-1}), respectively. Means ± SD are reported in the right graphs and are the results of three separate experiments for NF cells and of eight separate experiments for both ICM and DCM cells (* = $P < 0.05$ *vs.* buffer at the Kruskal–Wallis test).

myocytes with JAK2 antagonism prevented Ang II-induced JAK2, STAT2 and STAT5 tyrosine phosphorylation (Fig. 4B). Ang II-induced JAK2, STAT2 and STAT5 tyrosine phosphorylation were also abolished by ROS inhibition, both in ICM and DCM failing cardiomyocytes (Fig. 4B). In human failing myocytes (DCM) Ang II induced Fas-L gene expression independently of glucose concentration (1.15 ± 0.27-fold and 1.28 ± 0.36-fold *vs.* buffer at 30 and 60 min, respectively; $P < 0.05$ for both) (Fig. 5A). Ang II-induced Fas-L response was abolished by p38MAPK inhibition, being unaffected by JAK2, or ERK1/2 antagonism (Fig. 5B). Ang II stimulation failed to enhance Bcl-xL and IRF-1 genes expression in human failing myocytes (Fig. 5A and B).

Conclusion

Present findings reveal that (1) in non-failing cardiomyocytes Ang II induced JAK2 activation is followed by STAT3 activation and Bcl-xL gene overexpression, (2) the gene response being mediated by a cross-link between ERK1/2 and JAK2, whereas (3) in failing human cardiomyocytes Ang II-induced STAT3 and Bcl-xL gene overexpression are abolished, being replaced by STAT2 and STAT5 activation. Ang II-induced Fas-L gene overexpression seems to be mediated by a JAK2/STAT independent pathway (p38MAPK).

In cardiac fibroblasts, Ang II-stimulated generation of ROS[17] directly allows the activation of a ROS-sensitive tyrosine kinase, JAK2.[18] Differently from fibroblasts,[19] non-failing adult cardiomyocytes require the presence of high glucose levels, causing a marked increase of NADPH oxidase activity,[20] to allow Ang II-mediated JAK2 activation.[8] A similar effect was also observed in mesangial cells.[21]

In non-failing myocytes STAT3 activation was inhibited by JAK2 antagonism. Although STAT3 activation is usually associated with pro-hypertrophic and cardioprotective response,[22] information linking STAT3 to specific target genes in adult cardiomyocytes is limited. Data obtained from a mouse embryonic fibroblast cell line (NIH3T3 cells) indicated Fas-L

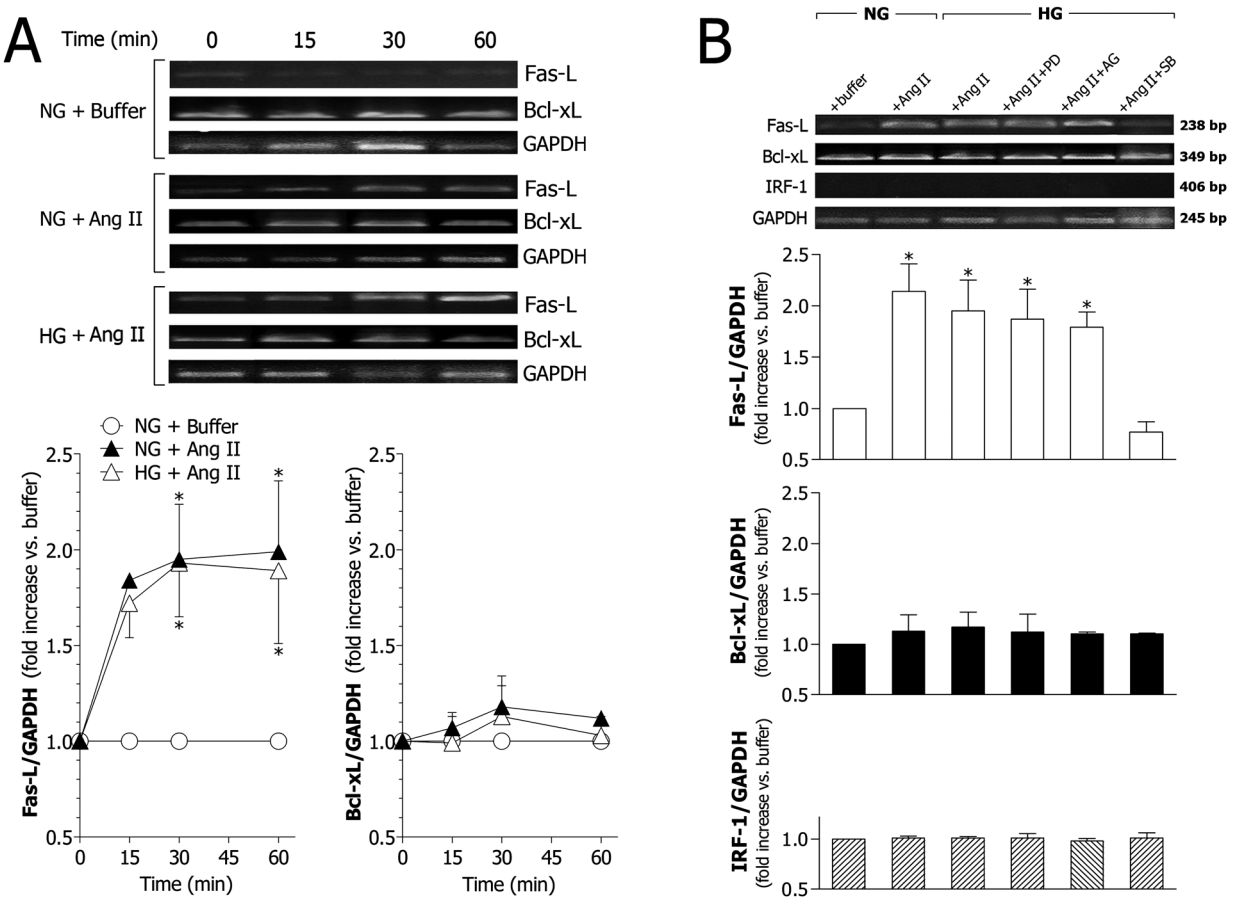

Fig. 5 In failing human cardiomyocytes Ang II-induced Fas-L mRNA overexpression occurs independently of ERK1/2 and JAK2 activation, being inhibited by p38MAPK antagonism. No responses of Bcl-xL or IRF-1 genes were observed. (A) Upper panels: representative RT-PCR experiments of Fas-L, Bcl-xL, and GAPDH mRNA expression in human failing myocytes (DCM) at baseline and after 15, 30, and 60 min of stimulation with buffer or Ang II (100 nM) in the presence of normal (NG, 5 mM), or high glucose (HG, 25 mM) concentrations (2 h of pre-incubation with glucose). Lower graphs: time course of genes (Fas-L and Bcl-xL)/GAPDH mRNA ratio expressed as fold increase relative to the buffer, set at 1.0. Data are means ± SD of four separate experiments (* = $P < 0.05$ *vs.* buffer at the Tukey *t* test). (B) Upper panels: representative RT-PCR experiments of Fas-L, Bcl-xL, IRF-1, and GAPDH mRNA expression in human failing myocytes (DCM) 30 min after stimulation with buffer or Ang II in the presence of NG or HG buffer. Effects of inhibition of ERK1/2 (PD, PD98059, 30 μM), JAK2 (AG, AG490, 10 μM), or p38MAPK (SB, SB203580, 10 μM) added 60 min before Ang II stimulation in HG buffer. Lower graphs: fold increase of Fas-L, Bcl-xL, and IRF-1 mRNA expression (gene/GAPDH ratio) induced by Ang II (30 min) relative to the buffer, set at 1.0. Data are means ± SD of four separate experiments (* = $P < 0.05$ *vs.* buffer at the Kruskal–Wallis test).

as a target gene for tyrosine-phosphorylated STAT3.[14] Direct identification of STATs target genes is however limited by the possible interaction with other intracellular pathways. Present data indeed indicate that at least in human ventricular myocytes, a major role in Fas-L gene expression is played by the p38MAPK pathway, rather than by JAK2/STAT3, stressing the importance of considering the cell type in the heart and the limitation to extend results to different tissue or cell lines. In neonatal cardiomyocytes, Fas-L overexpression was indeed reported to be activated by a JAK2 independent pathway mediated by p38MAPK serine phosphorylation of STAT1.[23,24] Our results do not exclude the possible participation of STAT1. Differently from Fas-L, Ang II-stimulated Bcl-xL gene expression was blunted by JAK2 inhibition. Bcl-xL gene overexpression was also inhibited by ERK1/2 antagonism. ERK1/2 antagonism however did not affect JAK2 or STAT3 tyrosine phosphorylation, so that ERK1/2 and JAK2 seem to cooperate to induce Bcl-xL gene expression. These observations might be in line with previous data obtained in human fibroblast cell lines indicating that full transcriptional activity of STAT3 requires both tyrosine and serine phosphorylation,[7] STAT3 being a better substrate for ERK1/2 than for p38MAPK.[25] Therefore, in human cardiomyocytes, as well as in fibroblasts, Bcl-xL seems to be linked to STAT3, the transcriptional activity of STAT3 being enforced by ERK1/2. The observed STAT3 activation induced by Ang II in the presence of high glucose may have relevant implications. Glucose concentrations used in *in vitro* study can be reached *in vivo* so that the possible participation of this mechanism in diabetic cardiomyopathy can be hypothesized.

The downstream STATs response of failing myocytes is peculiar because Ang II-induced JAK2 activation, which occurs independently of glucose concentration, is not followed by the STAT3 phosphorylation observed in non-failing cells. The impaired STAT3 response is consistent with the reduced number of STAT3-positive cardiomyocytes observed in left

ventricle preparations of human failing hearts.[2] The combined alterations of STAT3 and ERK1/2 activation observed in failing human ventricular myocytes might be responsible for the altered Bcl-xL response.

The functional meaning of STAT2 and STAT5 Ang II-mediated activation in human failing cardiomyocytes needs further investigations, although an involvement of up-regulation of pro-inflammatory pathways can be hypothesized. STAT2 is the member of the family related to the interferon (IFN)-like responses,[26] and it may be surmised that STAT2 activation contributes to the myocardial inflammation present in the advanced heart failure. The activation of STAT5 by Ang II might promote an autocrine loop for the maintenance of cardiac Ang II generation since angiotensinogen gene promoter in myocytes is a target of activated STAT5.[27]

Limitations of our study need to be considered. Notwithstanding the value of obtaining present data in isolated ventricular myocytes rather than in homogenated hearts, the number of subjects investigated was low, due to the particularly limited availability of non-failing human cardiomyocytes. We can also consider the possibility that Ang II antagonists and beta-blockers assumed by patients might have played a role in the observed altered signaling response in human left ventricular cardiomyocytes. However, previous studies reported that the assumption of prescribed drugs had no residual effect on Ang II-induced gene expression,[16] JAK2 phosphorylation,[8] or ERK1/2 activation,[9] after cardiomyocytes isolation. In a very recent paper Vergara *et al.*[28] demonstrated that the signalling response triggered by ERK1/2 is down-regulated in ovarian cancer cells after treatment with a phytochemical involved in many cellular signalling pathways indicating a possible evidence of the potential protective action of antioxidant molecules. Experimental studies also provided insights regarding the role played by ROS overproduction in the cardiac dysfunction, so further studies will be needed to fully clarify the role of ROS generation in failing human cardiomyocytes. From a clinical point of view, conflicting results of randomized placebo-controlled trials investigating the effects of antioxidants therapies challenge researchers to evaluate the ideal study patients to study, and the appropriate trial duration.[29,30]

Finally, the present study can be considered mainly observational because the mechanisms responsible for the altered STATs response in human cardiomyocytes in failing hearts still remain to be clarified.

To conclude, even with limitations, our results may have relevant clinical implications because the onset of an impaired STAT3 response may further facilitate the progression to failure.

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