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Cardiac resynchronization therapy-induced left ventricular reverse remodelling is associated with reduced plasma annexin A5

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Aims Cardiac resynchronization therapy (CRT) diminishes cardiac apoptosis and improves systolic function in heart failure (HF) patients with ventricular dyssynchrony. Plasma annexin A5 (AnxA5), a protein related to cellular damage, is associated with systolic dysfunction. We investigated whether the response to CRT is associated with plasma AnxA5. We also studied AnxA5 overexpression effects in HL-1 cardiomyocytes.

Methods and results AnxA5 ELISA was performed in plasma from 57 patients with HF and ventricular dyssynchrony at baseline and after 1 year of CRT. Patients were categorized as responders if they presented both a reduction in left ventricular (LV) end-systolic volume index (LVESVi) >10% and an increase in LV ejection fraction (LVEF) >10%. HL-1 cells were transfected with human AnxA5 cDNA, and AnxA5, PKC, Akt, p38MAPK, Bcl-2, mitochondrial integrity, caspase-3, and ATP were assessed. At baseline, an increased plasma AnxA5 level was associated with decreased LVEF and increased LVEDVi values ($P < 0.05$). No differences in baseline AnxA5 were observed between responders and non-responders. After CRT, AnxA5 decreased ($P = 0.001$) in responders but remained unchanged in non-responders. Final values of AnxA5 were independently associated with LVEF ($r = -0.387$, $P = 0.003$) and LVESVi ($r = 0.403$, $P = 0.004$) in all patients. Compared with control cells, AnxA5-transfected cells exhibited AnxA5 overexpression, decreased PKC and Akt and increased p38MAPK and Bcl-2 phosphorylation, loss of mitochondrial integrity, caspase-3 activation, and decreased ATP.

Conclusion CRT-induced LV reverse remodelling is associated with reduction in plasma AnxA5. The excess of AnxA5 is detrimental for HL-1 cardiomyocytes. Collectively, these data suggest that the beneficial effects of CRT might be related to an AnxA5 decrease.

Keywords Annexin A5 • Mitochondrial dysfunction • Heart failure • Resynchronization • Ventricular dyssynchrony

1. Introduction

Cardiac resynchronization therapy (CRT) is an effective treatment to reverse left ventricular (LV) remodelling and enhance systolic function while improving long-term outcome and survival in patients with congestive heart failure (HF) and ventricular dyssynchrony.[1,2] Although most patients with HF may benefit from CRT, 30% of patients do not respond clinically to CRT and up to 45% do not show evidence of reverse LV remodelling.[3–5] Asynchronous ventricular activation causes changes in myocardial tissue composition, including cardiomyocyte loss resulting from enhanced apoptosis, likely due to an excess of pro-apoptotic molecules.[6] Interestingly, it has been demonstrated that the beneficial clinical effects of CRT are associated with reduction in cardiac apoptosis in patients[7] and animals[8] with HF and ventricular dyssynchrony.

Annexin A5 (AnxA5) is a 35 kDa plasma protein, with a high affinity for phosphatidylserine in the nanomolar range.[9] Recent *in vitro*[10] and *in vivo*[11] experimental data suggest that AnxA5 may be involved in the stimulation of cardiomyocyte apoptosis during cardiac pathological conditions. Specifically, AnxA5 has been suggested to affect

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mitochondrial permeability and function.[12] In addition, AnxA5 has been shown to inhibit PKC activity,[13] an effect that initiates apoptosis in a variety of cell types.[14] Increased amounts of AnxA5 have been reported in the myocardium[15–17] and plasma[17] of HF patients. Of interest, inverse correlations were found between both myocardial and plasma AnxA5 and LV ejection fraction (LVEF) and volumes in these patients.[17]

We thus have hypothesized that long-term response to CRT, as assessed in terms of LV reverse remodelling, should be associated with a reduction in circulating AnxA5. To test this hypothesis, plasma AnxA5 was measured in patients with HF and ventricular dyssynchrony before and after 1 year of CRT. In addition, to further explore the potential effects of AnxA5 on cellular damage, we performed *in vitro* studies to analyse the effects of AnxA5 overexpression on phosphorylation of survival and stress protein kinases, inactivation of Bcl-2 by phosphorylation at Ser87,[18] mitochondrial integrity, proteolytic activation, and energy availability (i.e. ATP), in cultured murine HL-1 cardiomyocytes.

2. Methods

An expanded methods section is available in Supplementary material online

2.1. Clinical studies

2.1.1 Study design and subjects

All subjects gave written informed consent to participate in the study, and the Ethics Committee of the University Clinic of Navarra on human research approved the study protocol. The study conformed to the principles of the Declaration of Helsinki.

Between September 2005 and July 2007, 61 consecutive patients scheduled for CRT with HF in New York Heart Association (NYHA) functional class III or IV despite optimal pharmacologic therapy, LVEF $<35\%$, and left bundle branch block with a QRS duration >130 ms were screened for this study. Individuals with atrial fibrillation or an indication for implantation of a cardioverter-defibrillator were also included in the study. In patients with permanent atrial fibrillation, biventricular pacing was ensured with radiofrequency ablation of the AV junction or drug therapy to obtain permanent ($>80\%$) biventricular pacing.

Evaluation of patients at baseline (pre-implant) and at the 1-year follow-up included NYHA functional class, quality-of-life evaluation (with the use of the Minnesota Living with Heart Failure Questionaire), a standardized 6-min walk test, an echocardiographic study and obtention of blood samples for biochemical determinations. At 1 year, patients were categorized as responders if they exhibited LV reverse remodelling, defined by a reduction $>10\%$ in LV end-systolic volume index (LVESVi)[19] and an increment $>10\%$ in LVEF,[20] and as non-responders if they did not decrease LVESVi or increase LVEF at the end of the follow-up. If patients were submitted to cardiac transplantation before the 12-month follow-up showing no signs of response in the echochardiographic examination, they were considered as non-responders.

A group of 15 healthy subjects (11 men and 4 women; mean age, 65 ± 1.9; range 49–74 years) recruited at the University Clinic were used as control subjects for biochemical studies. None of these subjects exhibited abnormalities in the echocardiographic examination.

2.1.2 Device implantation and echocardiographic evaluation

Device implantation was performed as previously described.[21] Transthoracic two-dimensional echocardiograms, M-mode recordings, and Doppler ultrasound measurements were performed in each patient at baseline and 1 year thereafter using a Sonos 5500 ultrasound system (Phillips) as previously described.[21] For details, see Supplementary material online.

2.1.3 Blood sampling and biochemical determination

Blood samples were withdrawn from the left antecubital vein at the time of the clinical studies and stored at -20°C. Plasma AnxA5 was measured by using an AnxA5-specific ELISA (Zymutest Annexin V, Hyphen BioMed) as previously described.[17] The inter-assay and intra-assay variations for determining AnxA5 were 6 and 2.4%, respectively. The sensitivity was 0.1 ng/mL.

2.2 Experimental studies

2.2.1 HL-1 cell culture

HL-1 murine cells were a gift from Dr. William C Claycomb (Louisiana State University Health Sciences Center, New Orleans, LA). They were cultured in Claycomb medium supplemented with 10% foetal bovine serum, 1% penicillin/streptomycin, 1% norepinephrine and 1% L-glutamine, in a 5% CO_2 humified atmosphere at 37°C.

2.2.2. Construction of recombinant vectors

Complementary DNA (cDNA) of human AnxA5 was obtained by RT-PCR by using specific primers. PCR products were purified, cloned into a pcDNA3.1/V5-His© TOPO® vector and transformed into TOP10 *E. coli* cells. The recombinant vector obtained from these cells was sequenced showing the predicted sequence of human AnxA5. For details, see Supplementary material online.

2.2.3 Transfection of human AnxA5 in HL-1 cardiomyocytes

All transfections were performed with a mixture of human AnxA5 expression vector (50 and 100 ng) and β-galactosidase expression vector as control for transfection efficiency (500 ng) in HL-1 cardiomyocytes using a standard protocol as previously described (15). Cells were transfected at 40–50% confluence using the Lipofectamine 2000 reagent (Invitrogen) and OPTIMEM (GIBCO). Cell viability of transfected cells in all experimental conditions was determined by flow cytometry detection of AnxA5 and propidium iodide staining and MTT assay (see more details in Supplementary material online).

2.2.4 Protein extraction and subcellular fractioning

Total protein, cytosol and mitochondrial-enriched fractions were obtained from HL-1 cardiomyocytes as detailed in Supplementary material online.

2.2.5 Western blot studies

Human AnxA5, PKC, PCK-P, Akt, Akt-P (Ser473), p38MAPK, p38MAPK-P (Thr180/Tyr182), Bcl-2, and Bcl-2-P (Ser87) expression were analysed by western blot as detailed in Supplementary material online.

2.2.6 Extracellular AnxA5 protein quantification

AnxA5 antigen was measured in the extracellular medium from HL-1 cardiomyocytes transfected with human AnxA5 by using an AnxA5-specific ELISA (Zymutest Annexin V, Hyphen BioMed) as described previously.[17]

2.2.7 Analysis of mitochondrial damage and caspase-3 protease activation

Depolarization of the mitochondrial membrane was analysed as the ratio JC1 monomers (527 nm)/JC1 aggregates (590 nm) by flow cytometry as previously described (15). In addition, cytochrome c quantification and caspase-3 activation was determined by Western blot as described in online Supplementary material.

2.2.8 Quantification of intracellular ATP

The amount of intracellular ATP in HL-1 cardiomyocytes was quantified by using a commercial kit (ATP Bioluminescence Assay Kit CLS II, Roche).

2.2.9 Statistical analysis

Differences at baseline between subgroups and differences at baseline and after 1 year of CRT between responders and non-responders were tested by Student's *t*-test for unpaired data once normality was demonstrated (Shapiro–Wilks test); otherwise, a non-parametric test (Mann–Whitney *U*-test) was used. Differences in AnxA5 values between the two groups of patients at baseline and the control group were tested by one-way ANOVA followed by a Student–Newman–Keuls test once normality was checked (Shapiro–Wilks test); otherwise, the non-parametric Kruskal–Wallis test followed by a Mann–Whitney *U* test (adjusting the α-level by Bonferroni inequality) was used. Differences in parameters before and after treatment within each group of patients were tested by the student's t-test for paired data once normality was demonstrated (Shapiro–Wilks test); otherwise, a non-parametric test (Wilcoxon test) was used. Categorical variables were analysed by the χ^2 test or Fisher's exact test when necessary. Correlations were estimated by univariate regression analysis using Pearson correlation coefficient once normality was demonstrated (Shapiro–Wilks test) (non-parametric distributed variables were examined after logarithmic transformation); otherwise, Spearman correlation coefficient was used. Multivariate linear regression models were used to assess the independent relationship between the variable of interest (plasma levels of AnxA5) and LVEF, LVESVi and LVEDVi, after adjustment for relevant covariates (age, gender, functional class and pharmacological treatments). Differences among *in vitro* conditions were tested by 1-way ANOVA followed by a Student–Newman–Keuls test once normality was checked (Shapiro–Wilks test); otherwise, the non-parametric Kruskal-Wallis test followed by a Mann–Whitney *U* test (adjusting the α–level by Bonferroni inequality) was used. Variables are expressed as mean ± SEM and 95% confidence interval (clinical studies) or mean ± SEM (experimental studies) and categorical variables as numbers and percentages. Statistical significance was defined as two-sided $P < 0.05$. The analysis were performed using the program SPSS (15.0 version)

3. Results

3.1. Clinical findings

3.1.1 Classification of patients and baseline characteristics

At the end of follow-up, 31 patients (51%) were considered responders to CRT according to the predefined criteria. There were 30 non-responders (49%), of whom 4 were submitted to heart transplantation during the study. None of the patients died before the end of the study.

Baseline clinical and echocardiographic characteristics of the patients in each group are presented in *Table 1*. Most patients in the two groups were treated with the combination of a loop diuretic, a beta-blocker, and either an angiotensin-converting enzyme inhibitor or an angiotensin II type 1 receptor blocker. No differences were found between the two groups in the distribution of the different classes of pharmacological compounds.

After analysing the histogram frequency distribution of plasma AnxA5 data at baseline in all patients we have observed two frequency patterns in our population differentiated by an AnxA5 value in plasma of 24 ng/mL. According to this value, we have categorized patients at baseline into two groups: patients with plasma AnxA5 >24 ng/mL or patients with plasma AnxA5 levels <24 ng/mL. As shown in *Figure 1*, patients exhibiting plasma AnxA5 >24 ng/mL showed lower values of LVEF (panel A) and higher values of LVESVi (panel B) ($P < 0.05$) as compared with patients showing AnxA5 plasma values <24 ng/mL [LVEF: 23 ± 1.1 (95% confidence interval, CI: 20.9–25.5) vs. 26.3 ± 0.9 (95% CI: 24.5–28) %; LVESVi: 102 ± 8.5 (95% CI: 83.5–121) vs. 79.9 ± 4.5 (95% CI: 70.7–89.1) mL/m^2] (*Figure 1*).

No differences in the baseline values of AnxA5 were observed between the group of responders [17.4 ± 3.1 (95% CI: 11–24) ng/mL], and the group of non-responders [20 ± 3.4 (95% CI: 13–27) ng/mL]. The two groups of patients exhibited higher ($P < 0.0001$) AnxA5 values than in the control group [3.4 ± 0.5 (95% CI: 2.2–4.5) ng/mL].

3.1.2 Effects of CRT

As shown in *Table 1*, the distance walked in 6 min and the mean NYHA class improved ($P < 0.01$) after 1 year of CRT in both responders ($n = 31$) and non-responders that finished the study ($n = 26$). However, the improvement achieved when considering final values of these two parameters was higher ($P < 0.05$) in responders than in non-responders (*Table 1*).

Whereas the Tei index decreased ($P < 0.001$) in responders, it remained unchanged in non-responders (*Table 1*). Septal-to-lateral-wall motion delay (SLWMD) values decreased significantly in both groups of patients, although the final values of this parameter were lower ($P = 0.002$) in responders than in non-responders (*Table 1*).

Whereas LV diameters and volumes decreased ($P < 0.001$) in responders, they did not change in non-responders (*Table 1*). Thus, the final values of LV diameters and volume indexes were significantly lower in responders than in non-responders (*Table 1*). In addition, LVEF increased ($P < 0.001$) in responders, but remained unchanged in non-responders. Therefore, final values of LVEF were higher ($P < 0.001$) in responders than in non-responders (*Table 1*).

As shown in *Figure 2*, whereas AnxA5 plasma levels decreased ($P = 0.001$) after 1 year of CRT in the responder group [final value: 11.2 ± 2.3 (95% CI: 6.4–16) ng/mL], this parameter did not change in the non-responder group [final value: 17 ± 3.1 (95% CI: 11–23) ng/mL]. There were significant differences in the final values of these parameters between the two groups of patients ($P = 0.009$).

In the responder group, AnxA5 levels decreased at least one tertile in 58% of patients and remained in the same tertile in 42% of patients. In the non-responder group, AnxA5 levels decreased at least one tertile in 23% of patients, increased at least one tertile in 31% of patients and remained in the same tertile in 46% of patients. The differences between the two groups of patients were significant ($\chi^2 = 13.71$, $P = 0.001$).

3.1.3 Analysis of associations

After 1 year of CRT, continuous correlations between plasma AnxA5 and several echocardiographic parameters were found. As shown in *Figure 3*, plasma AnxA5 was inversely correlated with LVEF ($r = -0.387$, $P = 0.003$) and directly correlated with LVESVi ($r = 0.403$, $P = 0.004$) in all patients. Furthermore, there was a direct correlation between plasma AnxA5 and LVEDVi ($r = 0.423$, $P = 0.003$) in all patients. Multiple linear regression analysis showed that, when adjusted for confounding factors such as age, gender, functional class and treatment (angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, beta-blockers, spironolactone, diuretics, digoxin), the aforementioned associations remained significant (LVEF: β-coefficient = −0.364, $P = 0.020$; LVESVi: β-coefficient = 0.430, $P = 0.011$; LVEDVi: β-coefficient = 0.419, $P = 0.016$) in all patients.

No correlations were found between plasma AnxA5 and the distance walked in 6 min and Tei index.

Table 1 Effects of CRT in heart failure patients classified according to the response to CRT as defined in the text

Parameters	Responders			Non-responders			*P* baseline	*P* 1 year
	Baseline	1 year	*P*	Baseline	1 year	*P*		
Age (years)	71 ± 1.6 (68–74)			69 ± 1.8 (65–72)			0.268	
Male/female (%, *n*)	81/19, 25/6			88/12, 23/3			0.333	
NYHA class	3.1 ± 0.1 (2.9–3.4)	2.1 ± 0.1 (1.9–2.4)	<0.001	3.1 ± 0.1 (2.8–3.3)	2.6 ± 0.2 (2.3–2.9)	0.002	0.672	0.018
6-min walk test (m)	340 ± 17 (306–374)	460 ± 15 (428–491)	<0.001	341 ± 23 (294–388)	383 ± 29 (322–444)	0.002	0.962	0.026
Electrocardiographic data								
Permanent AF (%, *n*)	33, 10			24, 6			0.642	
PR interval (ms)	181 ± 8 (164–199)			208 ± 18 (168–249)			0.164	
QRS interval (ms)	169 ± 6 (158–180)			156 ± 8 (139–172)			0.161	
LBBB (%, *n*)	84, 26			62, 16			0.081	
Aetiology (%, *n*)								
Ischaemic	36, 11			61, 16				
Dilated	58, 18			35, 9			0.146	
Valvular	6, 2			4, 1				
Lateral lead position (%,n)	71, 22			77, 20			0.490	
Medical treatment (%, *n*)								
ACEIs/ARAs	100, 31			100, 26				
Beta-blockers	48, 15			35, 9			0.218	
Spironolactone	13, 4			15, 4			0.542	
Diuretics	100, 31			100, 26				
Digoxin	42, 13			50, 13			0.366	
Tei index	0.86 ± 0.06 (0.74–0.98)	0.38 ± 0.06 (0.26–0.50)	<0.001	0.77 ± 0.05 (0.66–0.89)	0.61 ± 0.09 (0.43–0.79)	0.179	0.272	0.085
SLWMD	114 ± 8.1 (97–130)	40.9 ± 4.7 (31.3–50.5)	<0.001	97.5 ± 6.8 (83.5–112)	66.7 ± 6.6 (53.1–80.4)	0.031	0.124	0.002
LVEDD (mm)	69 ± 1.4 (66.1–71.9)	59.7 ± 1.4 (56.9–62.5)	<0.001	70.8 ± 2.1 (66.3–75.2)	70.2 ± 1.7 (66.6–73.8)	0.877	0.489	<0.001
LVESD (mm)	56.5 ± 1.6 (53.3–57.7)	45.4 ± 1.6 (42.2–48.6)	<0.001	58.4 ± 2.2 (53.9–62.8)	58 ± 1.7 (54.4–61.6)	0.839	0.466	<0.001
LVEDVi (mL/m^2)	116 ± 8 (99–133)	94.7 ± 6 (82.2–107)	<0.001	125 ± 8 (109–142)	122 ± 7 (107–137)	0.217	0.468	0.008
LVESVi (mL/m^2)	85.9 ± 6.1 (73.2–98.5)	58.7 ± 5 (48.4–68.9)	<0.001	92.5 ± 6.5 (79.1–106)	87.2 ± 6.4 (73.9–101)	0.172	0.500	0.001
LVEF (%)	26.1 ± 1 (24–28.2)	40.1 ± 1.3 (37.4–42.7)	<0.001	24.3 ± 1 (22.2–26.4)	24.4 ± 1.5 (21.4–27.5)	0.637	0.089	<0.001

Values are expressed as mean ± SEM and 95% confidence interval and categorical variables as numbers and percentages.
NYHA, New York Heart Association; AF, atrial fibrillation; LBBB, left bundle branch block; ACEIs, angiotensin converting enzyme inhibitors; ARAs, angiotensin II type 1 receptor antagonists; SLWMD, septal-to-lateral-wall motion delay; LVEDD, left ventricular end-diastolic diameter; LVESD, LV end-systolic diameter; LVEDVi, left ventricular end-diastolic volume index; LVESVi, left ventricular end-systolic volume index; LVEF, LV ejection fraction

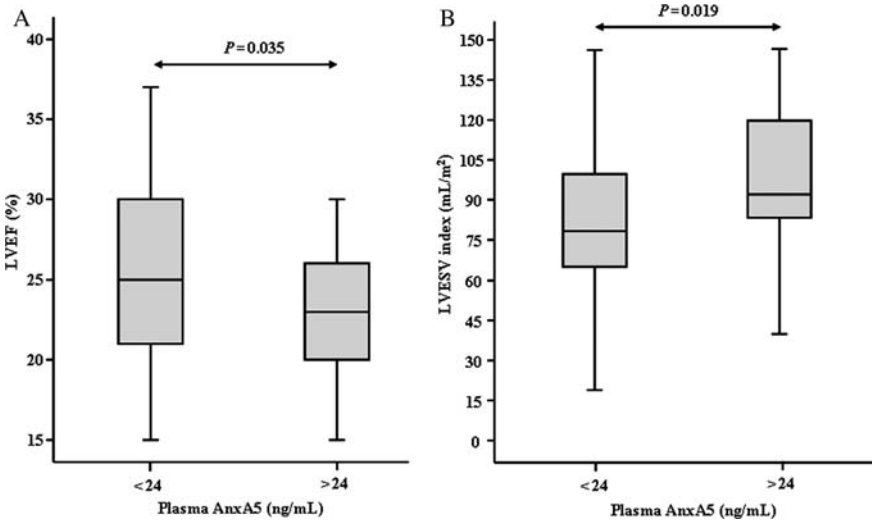

Figure 1 Distribution of systolic function parameters in HF patients with ventricular dyssynchrony categorized according to Annexin A5 (AnxA5) plasma levels of 24 ng/mL. Box plots show the 5th and 95th (vertical lines), 25th and 75th (boxes) and 50th (horizontal line) percentile values for LVEF (panel *A*) and LVESV index (panel *B*) in patients with HF and ventricular dyssynchrony at baseline.

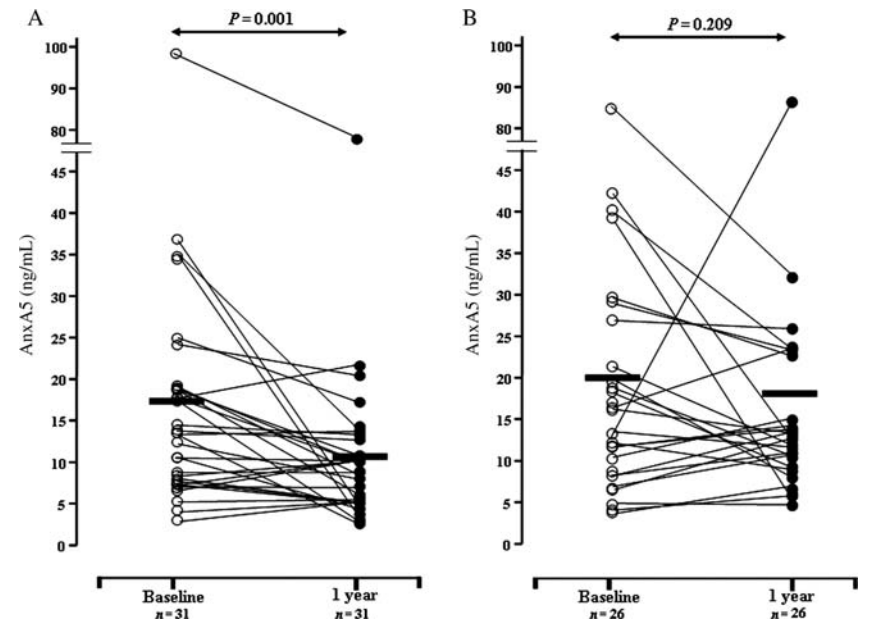

Figure 2 Annexin A5 (AnxA5) plasma concentration measured at baseline and after 1 year of cardiac resynchronization therapy in responder (panel *A*) and non-responder (panel *B*) patients. Bars indicate the average value of plasma AnxA5 concentration for each group.

3.2 Experimental findings

3.2.1 Expression of human AnxA5 protein

As shown in *Figure 4*, there was a dose-dependent increase in AnxA5 expression in total protein extracts (P for trend <0.001) and in the extracellular medium (P for trend <0.01) of AnxA5-transfected HL-1 cardiomyocytes. The increment in intracellular and extracellular AnxA5 was higher ($P < 0.05$) in HL-1 cells transfected with 100 ng of human AnxA5 cDNA than in control cells. Whereas the transfection methodology did not have major effects on HL-1 cell viability, this parameter was slightly reduced when cells were transfected with 100 ng of AnxA5 human cDNA as compared with control-transfected cells (*Figure S1*, online Supplementary material).

3.2.2 Analysis of phosphorylation of survival, stress-protein kinases, and Bcl-2

As observed in *Figure 5*, PKC phosphorylation was progressively inhibited (P for trend <0.0001) with the increase of AnxA5 expression in AnxA5-transfected HL-1 cells (*Figure 5A*). The inhibition in PKC activation was higher ($P < 0.001$) in HL-1 cardiomyocytes transfected with 100 ng human AnxA5 cDNA as compared with control cells.

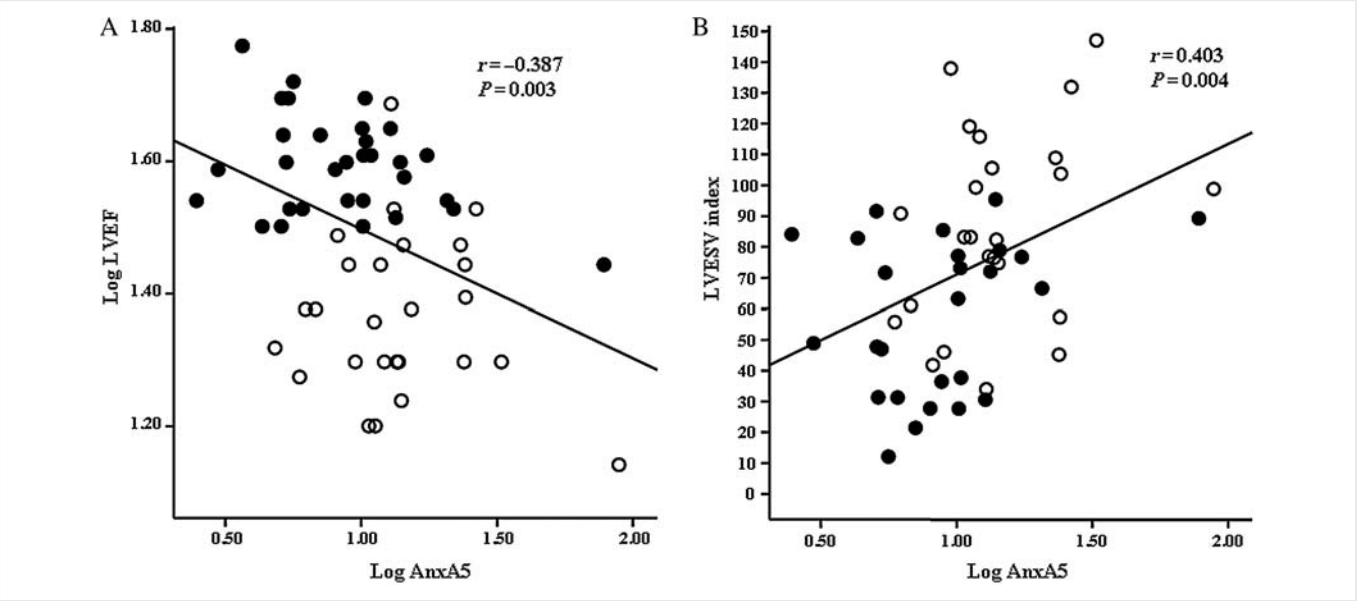

Figure 3 Associations between plasma Annexin A5 (AnxA5) with left ventricular ejection fraction (LVEF) (panel *A*) and left ventricular end-systolic volume index (LVESVi) (panel *B*) measured after 1 year of cardiac resynchronization therapy in responders (closed circles) and non-responders (open circles).

Akt phosphorylation at serine 473 was inhibited ($P < 0.05$) in cardiomyocytes transfected with 100 ng human AnxA5 cDNA as compared with control cells (*Figure 5B*).

As observed in *Figure 5C*, a progressive phosphorylation (*P* for trend <0.05) of p38 MAPK at Ser180 and Tyr182 in association with the progressive increase of human AnxA5 cDNA was observed in AnxA5-transfected HL-1 cells (*Figure 5C*).

Bcl-2 phosphorylation at S87 was enhanced ($P < 0.05$) in cardiomyocytes transfected with 100 ng human AnxA5 cDNA as compared with control cells (*Figure 5D*).

3.2.3 Mitochondrial damage and caspase-3 protease activation

A progressive depolarization of the mitochondrial membrane (*P* for trend <0.001) in association with the progressive increase of human AnxA5 cDNA was observed in AnxA5-transfected HL-1 cells (*Figure 6A*). The enhancement in mitochondrial membrane depolarization was higher ($P < 0.05$) in transfected HL-1 cells than in control cells.

As observed in *Figure 6B*, cytochrome c was progressively released from the mitochondria to the cytosol in transfected cells in a dose-dependent manner (*P* for trend <0.01). Cytochrome c release was increased ($P < 0.05$) in HL-1 cardiomyocytes transfected with 100 ng human AnxA5 cDNA as compared with control cells.

Caspase-3 activation was increased ($P < 0.05$) in HL-1 cardiomyocytes transfected with 50 and 100 ng of human AnxA5 as compared with control cells (*Figure 6C*).

3.2.4 ATP content

HL-1 cardiomyocytes transfected with human AnxA5 exhibited a dose-dependent decrease in the intracellular ATP content (*P* for trend <0.05) (*Figure 6D*). Compared with control cells, the decrease was significant ($P < 0.05$) in HL-1 cells transfected with 100 ng of human AnxA5.

4. Discussion

The major findings of the present study are the following: (i) plasma AnxA5 is associated with LV remodelling and dysfunction in HF patients with ventricular dyssynchony, (ii) CRT-induced reverse LV remodelling and improvement of systolic function is associated with reduction of plasma AnxA5, and (iii) AnxA5 overexpression is associated with inactivation and activation of survival and stress kinases, respectively, decreased Bcl-2 activation, increased depolarization and altered permeability of the mitochondrial membrane, caspase-3 protease activation and reduction of ATP availability in HL-1 cardiomyocytes.

Which is the origin of the excess of plasma AnxA5 in HF patients with ventricular dyssynchrony? Different causes for increased plasma AnxA5 levels have been described in the literature, such as the presence of sickle cell disease[22] and AnxA5 release from endothelial cells and platelets to the bloodstream after (traumatic) tissue injury.[23,24] In addition, AnxA5 plasma levels may also be influenced by chronic inflammation of the vessel wall as is the case in atherosclerosis.[25] Interestingly, several studies report that plasma AnxA5 levels are increased after myocardial infarction[24,26] or unstable angina.[26] In the cardiac context, and confirming previous findings by Song *et al.*[15] and Benevolensky *et al.*[16] we have observed that, in HF patients, there is an increased expression of AnxA5 in the myocardium, namely in cardiomyocytes. Moreover, we have demonstrated a gradient of the plasma concentration of AnxA5 from the coronary sinus blood to the antecubital vein blood suggesting that this protein is released from the heart through the coronary sinus.[17] In addition, the highly significant correlation observed between plasma concentration of AnxA5 in peripheral blood and coronary blood suggests that the heart is a major source of circulating AnxA5.[17] Furthermore, the strong direct correlations found between plasma and myocardial AnxA5 suggests that circulating AnxA5 may be a biomarker of myocardial AnxA5.[17] Therefore, it

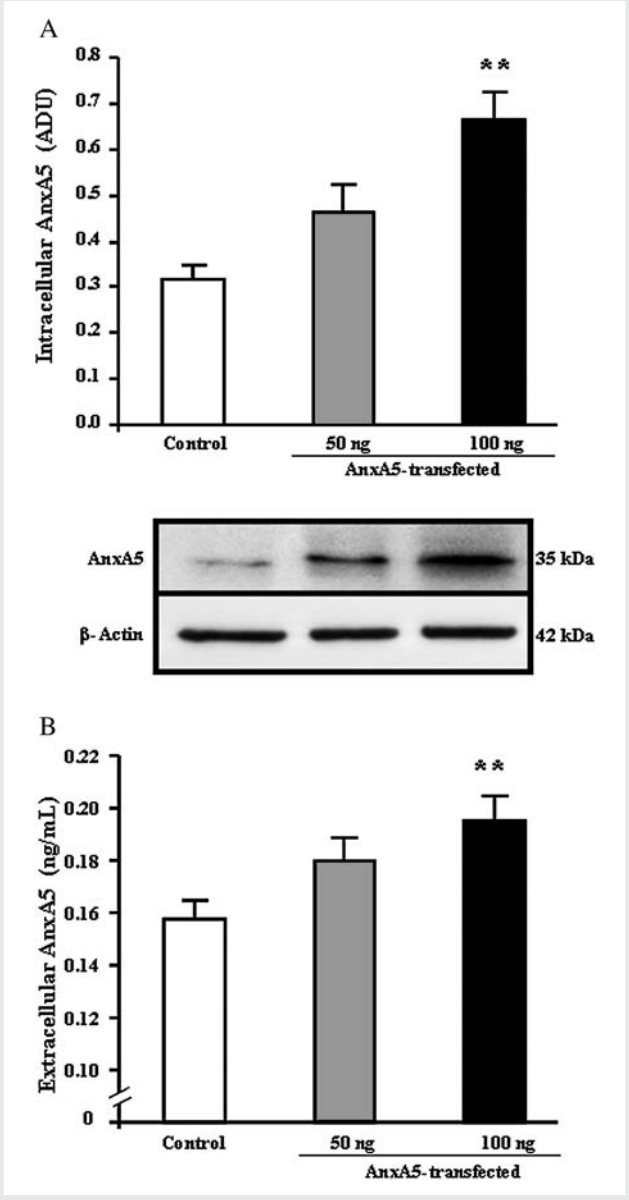

Figure 4 Intracellular (panel *A*) and extracellular (panel *B*) expression of annexin A5 (AnxA5) in HL-1 cardiomyocytes transfected with human AnxA5. Representative western blot autoradiograms of AnxA5 are presented in the bottom part of the panel *A*. Bars represent mean ± SEM ($n = 8$ to 10). $^{**}P < 0.01$ vs. control.

is likely that the excess of plasma AnxA5 in HF patients with ventricular dyssynchrony reflects an excess of myocardial AnxA5 and that reduction in plasma AnxA5 in patients who respond to CRT reflects the reduction in myocardial AnxA5. Then the question emerges on the mechanism(s) underlying the up-regulation of myocardial AnxA5 in conditions of ventricular dyssynchrony as well as the ability of CRT to reduce myocardial AnxA5 in responder patients. The possibility exists that CRT decreases the effects of overall mechanical stretch on cardiac cells associated with ventricular dyssynchrony and, in turn, reduces the stretch-induced up-regulation of AnxA5. Findings demonstrating that AnxA5 is stimulated by stretch in other non-cardiac cells are consistent with this possibility.[27,28]

Which is the potential role of an excess of AnxA5 in the failing heart of patients with ventricular dyssynchrony? Monceau *et al.*[10] have demonstrated that H_2O_2-induced apoptosis in rat cardiomyocytes is prevented by removing AnxA5 or blocking externalized AnxA5 by antibodies. The same group found that cardiomyocyte apoptosis during acute myocardial infarction in rats is related to early externalization of AnxA5 in the border zone.[11] It has been proposed that the pro-apoptotic effect of externalized AnxA5 in cardiomyocytes can be linked to Ca^{2+} channel activity and enhanced Ca^{2+} influx, as demonstrated in the case of chondrocytes.[29] In this regard, it has been demonstrated that DT40 cells lacking AnxA5 are resistant to Ca^{2+}-dependent apoptosis.[30] On the other hand, AnxA5 has been shown to inhibit PKC activity,[13] which induces apoptosis in a variety of cell types.[14] Supporting the last observation, we have reported here that AnxA5 overexpression is associated with reduced PKC phosphorylation in HL-1 cardiomyocytes. Moreover, survival kinase Akt and stress kinase p38 MAPK, are less and more activated by phosphorylation, respectively, in AnxA5 overexpressing HL-1 cells. In this regard, it is known that p38 MAPK phosphorylates Bcl-2 at Ser87, therefore inhibiting its antiapoptotic activity;[18] coherently, we have observed that Bcl-2 phosphorylation at this residue is increased in AnxA5-overexpressing HL-1 cells. Furthermore, we have demonstrated that AnxA5 is associated with the loss of mitochondrial integrity and function, and with the activation of caspase-3 in HL-1 cardiomyocytes. Thus, our results allow us to speculate that CRT beneficial effects in HF patients may be due, at least in part, to its ability to reduce AnxA5 excess and therefore contribute to inhibit the activation of the apoptotic mechanisms in cardiomyocytes. However, further analysis from *in vivo* experimental models and larger clinical studies is necessary to confirm this issue. Nonetheless, recent data by D'Ascia *et al.*[7] demonstrating that cardiac apoptosis decreased in HF patients who responded to CRT support this possibility.

Alternatively, it has been suggested that AnxA5 may alter cardiomyocyte function and contribute to HF through other pathways. For instance, Camors *et al.*[31] found that AnxA5 was forming a complex with Na^+/Ca^{2+} exchanger in both non-failing and failing human hearts suggesting a role as a regulatory factor of Ca^{2+}-handling proteins. Additionally, AnxA5 could be involved in cardiac dysfunction via compromised cardiomyocyte energetics, as reduced ATP availability of the failing heart has been shown to contribute to impaired contractile reserve.[32] In support of this possibility we found that cardiomyocytes overexpressing AnxA5 exhibit reduced ATP content, likely due to uncoupling of oxidative phosphorylation secondary to altered mitochondrial permeability.[33]

4.1 Limitations

First, we are aware that this was a study involving a relatively small number of patients with heterogeneous aetiologies that may have influenced the results. Nevertheless, we performed a parallel study to analyse whether the presence of ischaemic or non-ischaemic aetiologies may influence the beneficial effects of CRT. As shown in *Table 1* (Supplementary material file) CRT induced a similar improvement in clinical parameters and in LV structure and function, both in ischaemic and non-ischaemic patients. Furthermore, no differences were found when comparing all the clinical and echocardiographic parameters studied after 1 year of treatment between the two groups of patients. Second, the *in vitro* experiments have been performed in HL-1 cardiomyocytes. Although

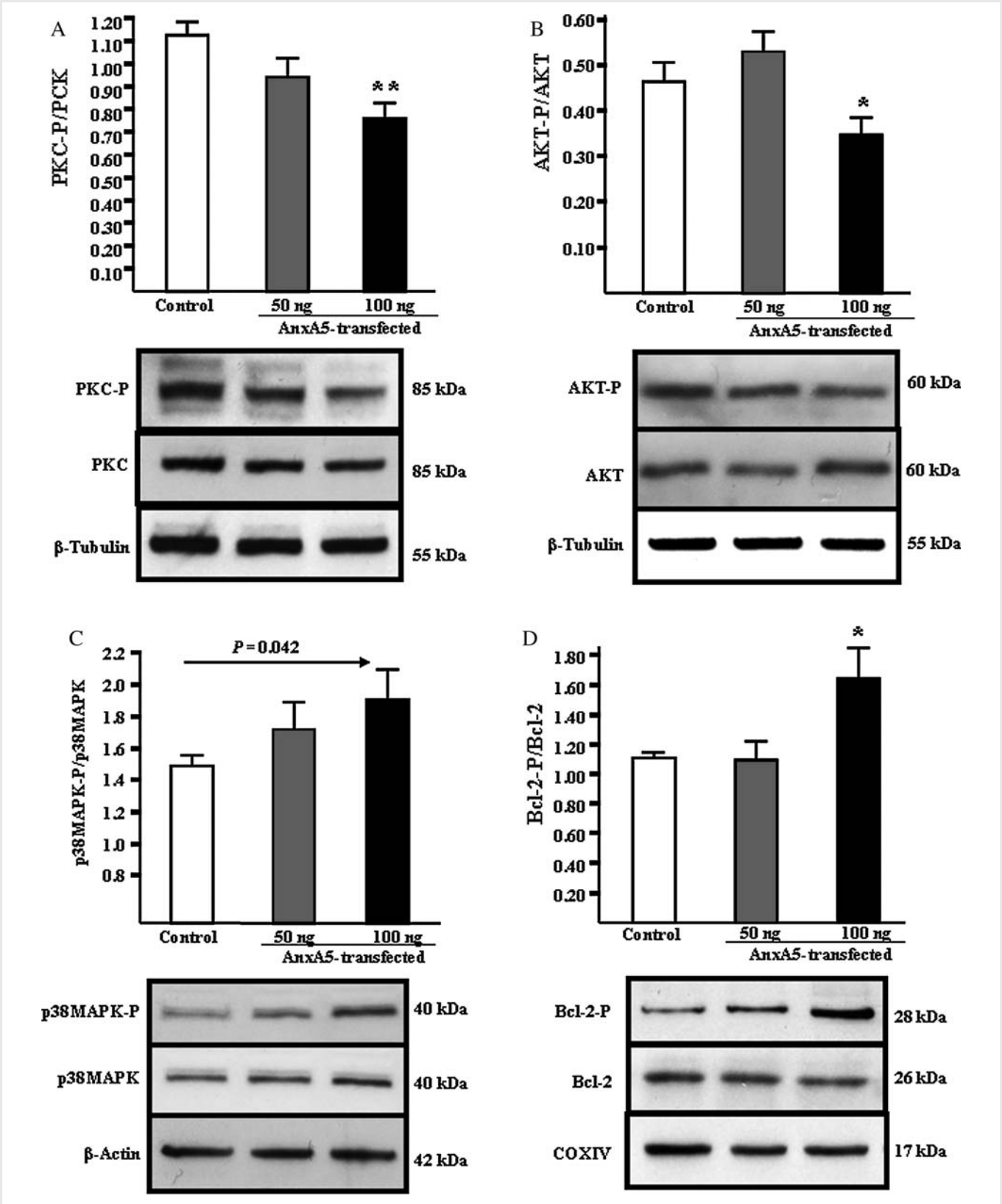

Figure 5 Ratio of phosphorylated PKC to non-phosphorylated PKC (panel *A*), ratio of phosphorylated Akt (Ser473) to non-phosphorylated Akt (panel *B*), ratio of phosphorylated p38 MAPK (Thr180/Tyr182) to non-phosphorylated p38 MAPK (panel *C*) and ratio of phosphorylated Bcl-2 (Ser87) to non-phosphorylated Bcl-2 (panel *D*) in HL-1 cells transfected with human annexin A5 (AnxA5). Representative Western blot autoradiograms are presented in the bottom part of each panel. Bars represent mean ± SEM ($n = 8–10$). *$P < 0.05$ vs. control, **$P < 0.01$ vs. control.

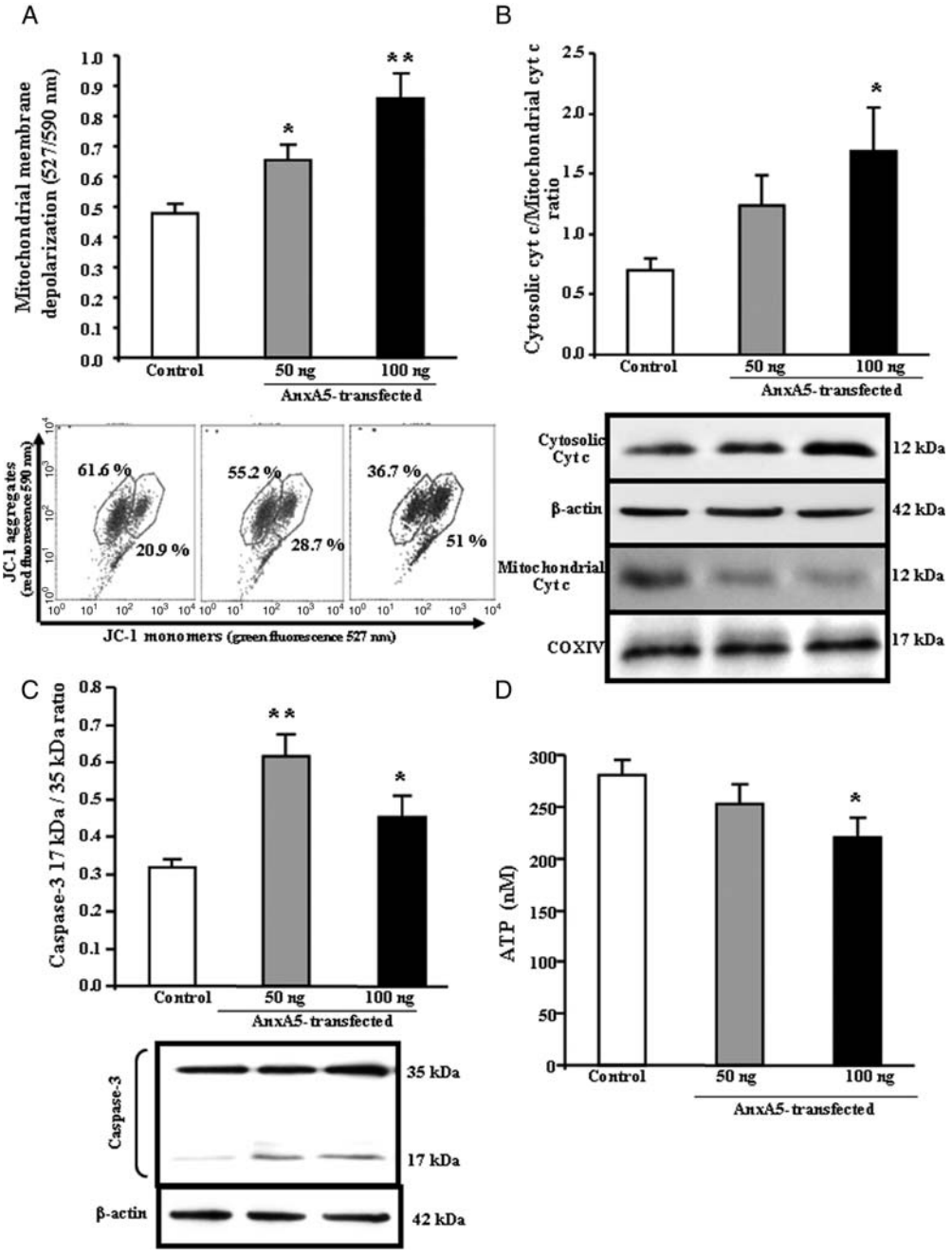

Figure 6 Depolarization of the mitochondrial membrane (panel *A*), cytosolic cytochrome c (cyt c)/mitochondrial cyt c ratio (Panel *B*), 17 kDa caspase-3/35 kDa caspase-3 ratio (Panel *C*) and intracellular ATP (Panel *D*) in HL-1 cells transfected with human annexin A5 (AnxA5). Representative graphics of flow cytometry determinations are presented in the bottom part of the panel *A*. Representative Western blot autoradiograms are presented in the bottom part of the panels *B* and *C*. Bars represent mean ± SEM ($n = 8–10$). *$P < 0.05$ vs. control, **$P < 0.01$ vs. control.

this is a cardiac muscle cell line derived from a mouse atrial cardiomyoyte tumour lineage, these cells maintain the ability to contract and retain differentiated cardiac morphological, biochemical, and electrophysiological properties characteristic of adult cardiomyocytes.[34]

In summary, the assessment of plasma AnxA5 provides information on one of the potential mechanisms contributing to the beneficial effects of CRT on LV structure and function in HF patients with ventricular dyssynchrony. Furthermore, this study suggests a role of AnxA5 as a potential mediator of mitochondrial damage and energetic compromise that may affect cardiomyocyte function. Thus, AnxA5 emerges as a potential target for therapies aimed to reverse LV remodelling and dysfunction in patients with ventricular dyssynchrony. Nonetheless, studies on experimental *in vivo* models and larger clinical prospective studies are required to definitively validate this approach.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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Conflict of interest: none declared

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