Upregulation of myocardial Annexin A5 in hypertensive heart disease: association with systolic dysfunction

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Aims To investigate whether Annexin A5 (AnxA5) is related to hypertensive heart disease (HHD) and whether this relation is dependent of apoptosis.

Methods and results Hypertensives without cardiac abnormalities (stage A), with left ventricular hypertrophy (LVH) (stage B), and with LVH and clinical manifestations of chronic HF (stage C), were studied. AnxA5 was quantified in endomyocardial biopsies by real time RT–PCR, Western blot, and immunohistochemistry, and apoptosis by DNA fragmentation, caspase-3 activation, and PARP and Bax/Bcl-2 ratios. Plasma AnxA5 was measured by ELISA in samples from the coronary sinus and the antecubital vein. Although AnxA5 mRNA did not change, myocardial and plasma AnxA5 were increased in hypertensives stages B and C compared with normotensives and hypertensives stage A. Myocardial AnxA5 was inversely correlated with parameters assessing systolic function in all hypertensives, this association being independent of apoptosis. Myocardial AnxA5 was directly correlated with plasma AnxA5. Plasma AnxA5 was inversely correlated with systolic function in all hypertensives.

Conclusion This cross-sectional study shows that myocardial AnxA5 upregulation is associated with HHD and impairment of systolic function in hypertensive patients, this association being independent of apoptosis. Plasma AnxA5 can be a marker of myocardial AnxA5 upregulation and systolic dysfunction in patients with HHD.

KEYWORDS
Annexin A5; Apoptosis; Heart failure; Hypertension

Introduction

Cardiomyocyte apoptosis has been shown to be abnormally stimulated in hypertensive heart disease (HHD), defined by the presence of left ventricular hypertrophy (LVH) in the absence of aortic stenosis and hypertrophic myocardial cardiomyopathy in hypertensive patients. Among the mechanisms linking cardiomyocyte apoptosis with deterioration of cardiac function and development of heart failure (HF), loss of cardiomyocytes due to cell death, compromise of oxidative phosphorylation and ATP production due to the loss of mitochondrial cytochrome C into the cytosol, and progressive contractile dysfunction of viable cardiomyocytes related to proteins involved in the apoptotic process have been proposed.

One of these proteins could be Annexin A5 (AnxA5). This 32–35 kDa Ca²⁺-binding protein has been shown to be upregulated, relocated to the sarcomere, and secreted during apoptosis activation. It has been proposed that overexpression and relocation of AnxA5 may contribute to the impairment of Ca²⁺ handling, which in turn may participate in the deterioration of systolic function in patients with HF.

We have hypothesized that an excess of myocardial AnxA5 may contribute to systolic dysfunction in patients HHD. To test this hypothesis, the present cross-sectional study was designed to analyse the relationship of AnxA5 with systolic function in patients with essential hypertension and LVH presenting with variable stages of HF. In addition, plasma levels of AnxA5 were analysed in the same patients to explore the potential usefulness of this protein as a marker of cardiac AnxA5 in hypertensive patients.

Methods

Subjects and clinical studies

All subjects gave written informed consent to participate in the study, and the locally appointed ethics committee approved the research protocol. The study complies with the Declaration of Helsinki.

The hypertensive population initially recruited in the study consisted of 105 Caucasian patients with systolic blood pressure and
diastolic blood pressure of more than 139 and 89 mmHg, respectively. They were recruited from 2005 to 2006 in the Division of Cardiology at the Donostia University Hospital, and their data were never included in previous studies. Ninety-seven patients had appropriate clinical and laboratory evaluation to exclude secondary hypertension. After complete medical examination, which included an echocardiographic examination and a diagnostic cardiac catheterization, 12 patients were excluded due to the presence of other cardiac diseases associated with myocardial apoptosis (i.e. aortic stenosis and coronary artery disease).

The remaining 85 patients were classified into three groups in accordance with the ACC/AHA Guidelines for the Evaluation and Management of Chronic HF in the adult.11 Twelve patients with no identified cardiac structural and functional abnormalities (stage A patients), 34 patients with LVH and no medical history of HF (stage B patients), and 39 patients with LVH and prior or current manifestations of HF and concurrent treatment for HF (stage C patients). The diagnosis of HF was made on a clinical basis by the presence of at least 1 major and 2 minor Framingham criteria.12 Furthermore, haemodynamic evidence of myocardial failure was obtained in each patient by measuring elevated left ventricular end-diastolic pressure and pulmonary wedge pressure (>12 mmHg in both cases).

A group of 18 normotensive subjects (12 men and 6 women; mean age, 58 ± 2 years; range 47–72 years) recruited in the Division of Cardiology at the Donostia University Hospital, were used as control subjects for molecular, histomorphological, and biochemical studies. They were subjects with repeated measurements of systolic and diastolic blood pressure below 140 and 90 mmHg, respectively. They presented with presumed coronary artery disease but were found to lack the disease at a coronary angiography. In addition, aortic stenosis was also excluded in these subjects after echocardiographic examination.

Three transvenous endomyocardial biopsies were taken as previously reported.13 One sample was employed for molecular studies, and the other two samples served for histomorphological and immunohistochemical studies.

Non-invasive cardiac studies

Two-dimensional echocardiographic imaging, targeted M-mode recordings, and Doppler ultrasound measurements were obtained in each patient. Left ventricular mass index (LVMI), left ventricular end-diastolic diameter (LVEDD), fractional shortening (FS), and ejection fraction (EF) were calculated as previously described.13 The presence of LVH was established when LVMI was >111 g/m² for men and >106 g/m² for women.14 In addition, we calculated the ratio of end-systolic wall stress to end-systolic volume index (ESS/ESVI), an index of systolic function that is thought to be independent of loading conditions,15 and used to assess left-ventricular chamber function.16 The following pulsed-Doppler measurements were obtained: maximum early and maximum late transmitral (when sinus rhythm) velocities in diastole, the deceleration time of the early mitral filling wave, and the isovolumetric relaxation time.

Western blot studies

Specific mouse monoclonal antibody for AnxA5 (Santa Cruz Technologies) at 1:100, for PARP (Cell Signalling) at 1/1000, for Bax (Santa Cruz Biotechnologies) at 1:500, and for Bcl-2 (Santa Cruz Biotechnologies) at 1:500 were incubated overnight at 4°C. Autoradiograms were analysed using an automatic densitometer (Bio-Rad, Molecular Imager FX). The blots were also probed with a monoclonal β-actin antibody (Sigma) as a control for loading. Data are expressed as arbitrary densitometric units (AU) relative to β-actin expression.

Reverse transcriptase–polymerase chain reaction studies

mRNA levels of AnxA5 gene were analysed by real-time quantitative RT–PCR. Reverse transcription was performed with 200 ng of total RNA, by using Superscript III reverse transcriptase (Invitrogen). Real-time PCR was performed with an ABI PRISM 7000 Sequence Detection System according to manufacturer’s recommendations (Applied Biosystems) by using specific TaqMan MGB fluorescent probes for human AnxA5 (Hs00174360_m1), and a specific TaqMan MGB fluorescent probe for human 18S ribosomal RNA, as endogenous control. Data are expressed as arbitrary units (AU) relative to constitutive 18S ribosomal RNA.

Histomorphological and immunohistochemical studies

AnxA5 was localized in 4% formaldehyde–fixed sections. Sections were covered with 10% normal donkey serum and incubated for 120 min. The slides were then incubated with a goat anti-AnxA5 antibody at 1:200 (Santa Cruz Biotechnology) followed by a Biotin-conjugated donkey anti-goat IgG at a dilution of 1:100 (Amersham), and stained with 3,3′-diaminobenzide tetrahydrochloride (DAB, Roche) and further counterstained with haematoxylin and eosin (H&E, Sigma). The myocardial surface area with positive staining for AnxA5 was assessed by quantitative morphometry with an automated image analysis system in the whole biopsy (AnalySYS, Soft Imaging System GmbH).

DNA fragmentation was assayed by the terminal deoxynucleotidyl transferase (TDT) reaction and the active caspase-3 immunostaining. DNA end-labeling (TUNEL) methodology was performed as previously described.2 The immunohistochemistry of the active form of caspase-3 was performed on myocardial sections parallel to those used for TUNEL staining and in accordance with a method previously described.7 To confirm caspase-3 activation, we analysed the formation of the 89 kDa fragment of the caspase substrate poly (ADP-ribose) polymerase (PARP) from its 116 kDa fragment.17 The ratio between the proapoptotic protein Bax and the anti-apoptotic protein Bcl-2 was assessed as an index of the mitochondrial apoptotic release.18

AnxA5 antigen quantification in plasma

AnxA5 antigen was measured in plasma from peripheral vein blood and coronary sinus blood by using an AnxA5-specific ELISA (Zymutest Annexin V, Hyphen BioMed) as described by Kraladisiri et al.19 The inter-assay and intra-assay variations for determining AnxA5 were 11.3 and 3.7%, respectively. The sensitivity (lower detection limit) was 0.1 ng/mL.

Statistical analysis

Variables are expressed as mean ± SD and categorical variables as numbers and percentages. To analyse the clinical differences between the normotensive group and the three groups of hypertensive patients, a 1-way ANOVA followed by a Student-Newman-Keuls test was performed 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. A linear test for trend was used to assess any tendency in the clinical and apoptotic parameters measured in normotensive subjects and hypertensive patients. The relation between variables was tested calculating Pearson correlation coefficient and, when applicable, Spearman correlation coefficient. Multivariable linear regression models were used to assess the independent relationship between the variable of interest (cardiac expression of AnxA5) and systolic function parameters (EF, FS, and ESS/ESVI) after adjustment for relevant covariates previously found to be associated with systolic function in univariable regression models: cardiomyocyte DNA fragmentation, PARP cleavage, and the ratio Bax/Bcl-2. The assumption of normality of residuals was satisfied for linear-regression analysis. Statistical significance was defined as two-sided P < 0.05. The analysis were performed using the program SPSS (13.0 version).
Results

Clinical characteristics of the patients

Table 1 shows the clinical characteristics of the three groups of hypertensive patients. None of stage A patients exhibited LVH or alterations in echocardiographic parameters assessing systolic and diastolic function. Twenty-seven (80%) stage B patients exhibited echocardiographic alterations suggestive of diastolic dysfunction and/or exhibited EF values >0.40 and <0.50. All stage C patients exhibited a depressed EF (<0.40). Most stage C patients were treated at baseline with the combination of a loop diuretic, a β-blocker, and either an angiotensin converting enzyme inhibitor (ACEI) or an angiotensin II type 1 receptor antagonist (ARA).

Assessment of apoptosis

As shown in Table 2, a trend (P < 0.01) towards progressive increment of DNA fragmentation, and higher 89 kDa PARP/116 kDa PARP and Bax/Bcl-2 ratios were observed from normotension to hypertension and through the different HF stages in hypertensive patients.

Hypertensive patients exhibited higher grades of caspase-3 immunostaining than normotensive subjects (χ² = 174.4, P < 0.001). Caspase-3 was predominantly located in cardiomyocytes and the staining was more intense in cells from stage C hypertensives than in cells from stages A and B hypertensives and normotensives (data not shown).

Cardiac expression and localization of AnxA5

AnxA5 expression was higher in the myocardium of stages B and C patients than in the myocardium of normotensive subjects and stage A patients (Figure 1). In addition, AnxA5 expression was increased in stage C hypertensives compared with the other two groups of hypertensives (Figure 1). No differences in this parameter were found between normotensives and stage A hypertensives.

No differences were found in AnxA5 mRNA between the three groups of patients (stage A: 54.83 ± 11.28 AU; stage B: 62.36 ± 14.62 AU; stage C: 60.98 ± 31.56 AU).

Whereas AnxA5 was found in the cytosol in cardiomyocytes from normotensive subjects (Figure 2A) and stage A hypertensives (Figure 2B), it was also found at the level of sarcolemma in hypertensives from stages B and C (Figures 2C and 2D, respectively). In addition, AnxA5 was found in intercalated disks (Figure 2E) and in the interstitial space (Figure 2F) in stage C hypertensives. Slight staining

<table>
<thead>
<tr>
<th>Parameters</th>
<th>NT</th>
<th>Stage A</th>
<th>Stage B</th>
<th>Stage C</th>
<th>P for trend</th>
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</thead>
<tbody>
<tr>
<td><strong>DNA fragmentation indexes (nuclei/10^2 nuclei)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Global</td>
<td>0.007 ± 0.008</td>
<td>0.033 ± 0.003</td>
<td>0.037 ± 0.019</td>
<td>0.064 ± 0.040</td>
<td>0.001</td>
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<tr>
<td>Cardiomyocytes</td>
<td>0.005 ± 0.004</td>
<td>0.027 ± 0.013</td>
<td>0.044 ± 0.029</td>
<td>0.113 ± 0.050</td>
<td>0.001</td>
</tr>
<tr>
<td>Non-cardiomyocytes</td>
<td>0.008 ± 0.009</td>
<td>0.033 ± 0.009</td>
<td>0.033 ± 0.012</td>
<td>0.042 ± 0.026</td>
<td>0.001</td>
</tr>
<tr>
<td>Bax/Bcl-2 ratio</td>
<td>0.74 ± 0.05</td>
<td>0.71 ± 0.4</td>
<td>1.00 ± 0.18</td>
<td>1.30 ± 0.76</td>
<td>0.007</td>
</tr>
</tbody>
</table>

NT, means normotensives; DNA fragmentation indexes represent TUNEL-positive nuclei/10^2 nuclei. PARP, means caspase substrate poly (ADP-ribose) polymerase. Values are expressed as mean ± SD.
for AnxA5 was also observed in endothelial cells (Figure 2G) and fibroblasts (Figure 2H) in stage C hypertensives, but not in the other groups of subjects.

**Plasma concentration of AnxA5**

AnxA5 immunoreactivity was detected in plasma from all the studied subjects. Plasma AnxA5 was increased in stages B and C patients compared with normotensive subjects and stage A patients both at the coronary sinus (Figure 3A) and at the antecubital vein (Figure 3B) level. Peripheral and coronary plasma AnxA5 were increased in stage C hypertensives compared with the other two groups of hypertensives. No differences in these parameters were found between normotensive and stage A hypertensives.

Whereas coronary AnxA5 was higher ($P < 0.01$) than peripheral AnxA5 in hypertensive patients ($19.97 \pm 3.89$ vs. $12.7 \pm 1.22$ ng/mL), no significant differences between these two parameters were observed in normotensive subjects ($5.66 \pm 3.02$ vs. $4.07 \pm 2.29$ ng/mL). In addition, there was a direct correlation ($r = 0.909, P < 0.001$) between coronary and peripheral AnxA5 in all hypertensive patients.

**Analysis of associations**

As shown in Table 3, inverse correlations were found among myocardial AnxA5 assessed by Western blot and immunocytochemistry and systolic function parameters (i.e. EF, FS, and ESS/ESVI). In addition, myocardial AnxA5 was directly correlated with LVEDD. A multiple linear regression analysis showed that, when adjusted for confounding factors assessing cardiac apoptosis (i.e. cardiomyocyte DNA fragmentation, PARP cleavage, and ratio Bax/Bcl-2), the inverse association between myocardial AnxA5 and systolic function remained significant (EF: $\beta$ coefficient $= -0.572$, $P = 0.043$; FS: $\beta$ coefficient $= -0.513$, $P = 0.043$; ESS/ESVI ratio: $\beta$ coefficient $= -0.632$, $P = 0.038$).

Furthermore, myocardial AnxA5 expression was directly correlated with plasma AnxA5 measured in coronary sinus ($r = 0.516, P = 0.001$) and antecubital vein ($r = 0.636, P = 0.001$) in all hypertensive patients. In addition, coronary
and peripheral plasma AnxA5 were inversely correlated with parameters of systolic function (i.e. EF, FS, and ESS/ESVI ratio) and directly with LVEDD in all hypertensive patients (Table 3).

Discussion

The main findings of this study are as follows: (i) AnxA5 is upregulated in the myocardium of hypertensive patients with LVH either without or with HF; (ii) an association exists between upregulation of myocardial AnxA5 and impairment of systolic function in hypertensive patients that is not influenced by apoptosis, and (iii) increased plasma AnxA5 is associated with enhanced myocardial AnxA5 and systolic dysfunction in hypertensive patients.

Pathophysiological meaning

The observation that AnxA5 is upregulated at the protein level but not at the mRNA level in the hypertrophied hypertensive myocardium suggests that this alteration is due to post-transcriptional modifications. Previous in vitro studies have shown post-transcriptional regulation of annexins as a function of cell cycle state. Thus, it is tempting to speculate that, as cell cycle activators, neurohumoral stimuli acting in the hypertensive patient, namely when HF is present, may influence AnxA5 post-transcriptional regulation.

Although it has been proposed that impairment of systolic function may ensue the loss of contractile mass due to complete death of cardiomyocytes, there is also evidence that apoptosis is often initiated but not always completed. Interrupted apoptosis can result in a subset of cardiomyocytes that have cytoplasmic protein loss, altered energetics, and contractile dysfunction. Finally, the apoptotic process may interfere with systolic function through alterations of intracellular Ca$^{2+}$. For instance, it has been suggested that the excess and strategic redistribution of AnxA5 towards sarcomeric structures and intercalated disks might be altering the handling of Ca$^{2+}$ in the heart, affecting the functional syncytium, and thus impairing contractility. In this study, a similar redistribution of AnxA5 was found in patients with HHD, specially in those presenting with HF. In addition, the excess of myocardial AnxA5 was associated with the impairment of systolic function (assessed by EF, FS, and the ESS/ESVI ratio). Of interest, this association remained significant when parameters assessing cardiac apoptosis are introduced as potential confounding factors. Thus, in patients with HHD, AnxA5 upregulation may play some role in the impairment of systolic function. In line with these data, different experimental studies show that a benzothiazepine derivative (JTV519 or K201) which binds to AnxA5 and blocks its ability to form Ca$^{2+}$ channels, improves myocardial contractility and preserves cardiac function.

Clinical application

Some findings reported here suggest the potential usefulness of AnxA5 measured in peripheral blood as a diagnostic

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Table 3  Associations found between Annexin A5 and parameters of cardiac function and morphology in all hypertensive patients

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Myocardial Annexin A5</th>
<th>Circulating Annexin A5</th>
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<tbody>
<tr>
<td></td>
<td>Staining r P</td>
<td>Expression r P</td>
</tr>
<tr>
<td>EF</td>
<td>$-0.748$ 0.001</td>
<td>$-0.431$ 0.001</td>
</tr>
<tr>
<td>FS</td>
<td>$-0.655$ 0.001</td>
<td>$-0.322$ 0.006</td>
</tr>
<tr>
<td>ESS/ESVI ratio</td>
<td>$-0.575$ 0.001</td>
<td>$-0.458$ 0.001</td>
</tr>
<tr>
<td>LVEDD</td>
<td>$0.474$ 0.001</td>
<td>$0.287$ 0.012</td>
</tr>
</tbody>
</table>

EF, ejection fraction; FS, fractional shortening; ESS, end-systolic wall stress; ESVI, end-systolic volume index; LVEDD, left ventricular end diastolic diameter.
marker of myocardial AnxA5 and systolic dysfunction in patients with HHD. First, we found that AnxA5 was located in the cardiac interstitium in stage C patients. It thus is likely that interstitial AnxA5 might reach the blood via cardiac lymph and/or venous drainage. Second, the finding that there is a gradient of AnxA5 from the coronary blood to the peripheral blood in hypertensive patients suggests that this protein is released from the heart through the coronary sinus. Furthermore, the highly significant correlation observed between peripheral AnxA5 and coronary AnxA5 suggests that the heart is, among other organs, an important source of circulating AnxA5 in hypertensive patients. Third, the associations found between plasma and myocardial AnxA5 suggest that circulating AnxA5 may be a marker of cardiac AnxA5 in hypertensive patients. Finally, the associations found between plasma AnxA5 and parameters assessing systolic function suggest that determination of this protein may also serve to assess mechanisms of systolic dysfunction potentially related to myocardial AnxA5.

Limitations

It must be recognized that therapy with different types of drugs may have confounded the findings and their interpretation. Nevertheless, one must consider that, since they are standard therapies for hypertension and HF, it is unreasonable to withdraw them for purposes of this investigation.

We performed biopsies of the right side of the interventricular septum to assess the structural effects of left ventricular pressure loading. However, previous studies have shown that this location may be representative of the apoptosis existing in the free wall of the hypertrophied human left ventricle.1

Although not only apoptotic, but also necrotic cells and cells undergoing DNA repair may be labelled by the TUNEL method, the associations found in this study between changes in DNA fragmentation and other parameters assessing apoptosis suggest that, beyond its real quantitative magnitude, cardiomyocyte apoptosis is highly increased in hypertensive patients, specially in stage C patients.

In conclusion, for the first time, we show that the upregulation of myocardial AnxA5 is associated with the impairment of left ventricular systolic function in HHD patients. Although descriptive in nature, our findings suggest that this association is not mediated by apoptosis. In addition, we show that the heart may be one of the sources of circulating AnxA5 in plasma in hypertensive patients. Thus, plasma AnxA5 may be useful as a biomarker of systolic dysfunction in patients with HHD. Nevertheless, because of the cross-sectional nature of this investigation, we are aware that further large non-invasive epidemiological studies and in vitro experiments are necessary to definitively validate this approach.

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Conflict of interest: none declared.

References

Clinical vignette

An epicardial electrode’s 8-year travel

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A 71-year-old patient was admitted to the cardiology department because of faintness and multiple ventricular premature beats. He had a coronary artery bypass surgery with sequential left internal mammary artery (LIMA) to left anterior descending artery, first and second diagonal branches in 1998. He had been asymptomatic until May 2006 when he had faintness during exertion with no chest discomfort. His physical examination and electrocardiogram were unremarkable.

Treadmill stress test showed multiple monomorphic premature ventricular beats without ST-depression. Coronary angiogram revealed a patent sequential LIMA graft with no additional significant stenosis. Transthoracic echocardiography revealed a linear hyper-echogenic image in the right heart chambers seen only from the subcostal view (Panels A and B). Sixty-four slice computed tomography showed a long curvilinear hyper-density image (600 HU) located in the right atrium and passing through the tricuspid valve into the right ventricle. The two edges of this foreign body were free from adjacent structures indicating that percutaneous removal would be possible (Panels C and D). Percutaneous retrieval was successful. The removed material was a ruptured epicardial electrode (Panel E).

To our knowledge, migration and percutaneous retrieval of epicardial electrode from intracavitary heart chambers have not yet been described. The mechanism of migration of this epicardial electrode to the right heart chambers is unclear. In our institution, temporary epicardial electrodes are removed or cut down in case of resistance at the seventh post-operative day. An intracavitary placement of the distal edge of the atrial epicardial electrode during cardiac surgery with the ruptured proximal edge progressively migrating to the right ventricle may be a possible explanation.

Panels A and B. Two-dimensional transthoracic echocardiogram subcostal four-chamber view depicting the presence of a foreign body (arrow) in the right heart chambers.

Panels C and D. Sixty-four slice computed tomography thin slice maximum intensity projection reconstruction image (Panel C) and volume rendering (Panel D) showed a long curvilinear hyper-density image (arrow) located in the right atrium and ventricle. RA, right atrium; RV, right ventricle; LA, left atrium; LV, left ventricle.

Panel E. The retrieved material was a 16 cm long epicardial electrode.