Proteomic Analysis of Early Left Ventricular Hypertrophy Secondary to Hypertension: Modulation by Antihypertensive Therapies

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Untreated or poorly controlled arterial hypertension induced the development of pathologic left ventricular hypertrophy (LVH), a common finding in hypertensive patients and a strong predictor of cardiovascular morbidity and mortality. The proteomic approach is a powerful technique to analyze a complex mixture of proteins in various settings. An experimental model of hypertension-induced early LVH was performed in spontaneously hypertensive rats, and the cardiac protein pattern compared with the normotensive Wistar Kyoto counterpart was analyzed. Fifteen altered protein spots were shown in the early stage of LVH. Compared with a previous animal model of established and regressed LVH, three protein spots were common in both models. These three altered protein spots corresponded to two unique proteins that were identified as Calsarcin-1 (CS-1) and ubiquinone biosynthesis protein COQ7 homolog. CS-1 is a negative regulator of the calcineurin/NF-AT pathway. Because upregulation in the expression levels of this protein was observed, the activation level of NF-κB by oxidative stress as an alternative pathway was investigated. It was found that antihypertensive therapies partially decreased oxidative stress and normalized the activation of NF-κB in the kidneys and aorta NF-κB activation but just moderately in the heart. This could be due to the interaction of any specific cardiac protein with any component of the NF-κB pathway. In this sense, CS-1 could be a good candidate because it is expressed preferentially in heart, to a lesser extent in smooth muscle cells, but not in kidney. Further investigations are necessary to elucidate the exact role of CS-1 and ubiquinone biosynthesis protein COQ7 in the setting of hypertension-induced LVH.


Arterial hypertension is the most frequent chronic disease in the developed world. It is a multisystemic disease that affects heart and kidney among other organs. The effects of untreated or poorly controlled systemic hypertension include both vascular dysfunction and the development of hypertensive heart disease with pathologic left ventricular hypertrophy (LVH). Initially, hypertrophy response is a compensatory mechanism that augments cardiac output, but sustained hypertrophy results in pathologic hypertrophy of cardiac muscle cells, which strongly predicts the development of various heart diseases and is a leading cause of cardiovascular morbidity and mortality worldwide (1,2). Multiple pathways and factors lead to pathologic LVH with a common final response that involves an increase in cell size and proteins synthesis, enhanced sarcomeric organization, and reexpression of fetal genes (3,4).

It has been shown that the proteomic approach is a useful technique to analyze a complex mixture of proteins in various settings, usually by combining two-dimensional electrophoresis (2-DE) and mass spectrometry (MS) (5). We recently analyzed the proteome of the established and regressed LVH. We found that established LVH showed a significant alteration in the cardiac protein profile compared with normal hearts, and those altered proteins were not fully normalized with antihypertensive therapies even after regression of the LVH (6). One of the altered proteins that remained unchanged despite the regression of the LVH was Calsarcin-1 (CS-1). This protein plays an important role as a negative regulator of calcineurin signaling (in striated muscle in vivo) and therefore in the activation of NF-AT transcription factor (7,8). Furthermore, CS-1 deficiency sensitizes the heart to hypertrophic stimuli (7). In our model of established LVH, we showed an upregulation of CS-1, suggesting the blockade of calcineurin/NF-AT pathway and hence the activation of another mechanism. It is well established that reactive oxygen species (ROS) function as second messenger signaling for the hypertrophy of cardiac myocytes through activation of NF-κB (9,10). We showed that animals with established LVH presented an important deregulation of many mitochondrial proteins that were just partially recovered
by antihypertensive therapies (6). The impairment in the mitochondrial respiratory function has been associated with generation of ROS and activation of the transcription factor NF-κB (11).

Our hypothesis is that proteins that remained altered along the progression of pathologic LVH could play key roles in the development of the disease and may be potential candidates for new antihypertensive drugs to prevent LVH. In this study, we first analyzed the proteome profile of an early stage of LVH in spontaneously hypertensive rats (SHR) versus normotensive Wistar Kyoto rats (WKY). Second, we searched common altered proteins in the early stage of LVH with the established or regressed LVH. Third, we checked the activation level of the transcription factor NF-κB as an alternative pathway to NF-AT in established and regressed LVH in the setting of hypertension.

Materials and Methods
Experimental Models and Drugs
Studies were performed with male SHR and their normotensive counterpart WKY (Crı́fı́a, Barcelona, Spain). Two stages of pathologic LVH, early and late/established, were studied. The first group consisted of six rats from both the control (WKY12) and SHR groups (SHR12) that were killed at 12 wk of age. A second group consisted of 12-wk-old SHR that spontaneously developed the disease for an additional 36 wk and were randomized to the following groups: (1) SHR without treatment (SHR48; n = 7); (2) SHR that were treated with 16 mg/kg per d quinapril (QUI; n = 7); and (3) SHR that received a combined therapy that consisted of 15 mg/kg per d doxazosin plus 1.6 mg/kg per d quinapril (DXQ; n = 6). WKY rats of the same age (WKY48) were studied as normotensive control (n = 7). These groups were killed at 48 wk of age.

All animals were allowed free water and food access in a controlled environment. Doxazosin and quinapril (as powdered hydrochloride salt) were provided by Pfizer (Barcelona, Spain) and given in the drinking water.

Weekly systolic BP (SBP) and cardiac hypertrophy were measured as described previously, as well as heart sample manipulation and histopathologic studies (6). The kidneys and aorta were perfused with cold sodium saline and quickly removed, and processed in a manner similar to subsequent MS protein analysis. Silver-stained 2-DE gels were scanned in a GS-800 calibrated densitometer (Quantity One; Bio-Rad, Hercules, CA) or a Bruker Ultraflex TOF/TOF MALDI (Bruker-Daltonics, Leipzig, Germany) mass spectrometers as described previously (6).

Measurement of 8-Iso-Prostaglandin F$_{2α}$
Serum levels of 8-iso-prostaglandin F$_{2α}$ (8-iso-PGF$_{2α}$) were measured using commercially available StressXpress 8-iso-PGF$_{2α}$ ELISA Kit (Stressgen Bioraagents, Victoria, BC, Canada) according to the manufacturer’s protocol.

Analysis of Transcription Factor Activity
NF-κB activity was evaluated by binding of 70 μg of protein extracts from renal or cardiac tissue with an oligo consensus labeled with γ$[^{32}$P]ATP, and the complexes formed were analyzed by electrophoretic mobility shift assay (EMSA). Nuclear protein extraction and EMSA were performed as described previously (14).

Southwestern Histochemistry
Paraffin-embedded aorta sections that were fixed in 0.5% paraformaldehyde were incubated with 50 pmol of digoxigenin-labeled NF-κB probe in buffer that contained 0.25% BSA and 1 μg/ml poly(dI-dC), followed by alkaline phosphatase-conjugated anti-digoxigenin IgG and colorimetric detection. Specificity was determined with mutant labeled probe and competition with 200-fold excess of unlabeled probe (15).

Statistical Analyses
Equality of variances was tested with the Levene test. Means of normally distributed continuous variables with equal variances were analyzed with t test or ANOVA. Otherwise, Mann-Whitney test and Kruskal-Wallis test were done. P < 0.05 was considered significant. Tests were done using the SPSS 11.5 software package (SPSS, Chicago, IL). Data are expressed as mean ± SEM. Proteomic statistical analysis was done using PD-Quest 7.1.1 software, which includes a statistical package. Spots were tested with t test.

Results
Hypertension-Induced LVH and Myocardial Injury
Twelve-week-old SHR (SHR12) showed a cardiac hypertrophy index (heart to body weight ratio ×10$^3$) that was significantly higher than that of the age-matched WKY controls (3.9 ± 0.1 versus 3.0 ± 0.1; P < 0.001). However, cardiac analysis of SHR12 showed neither fibrotic nor inflammatory foci significantly higher than the WKY12 (1 ± 1 versus 0 ± 0; NS).
Protein Expression Profile

Comparison between WKY12 and SHR12 was performed using the replicate group option and the statistic software package of PD-Quest 7.1.1. Spot quantification was normalized on the basis of the total staining density of the image to compensate for any variation in protein loading and development level of silver staining. The differential expression was calculated for every spot that could be matched in all of the samples of at least one group.

From all of the spots resolved in the pH 4 to 7 range by 2-DE of myocardial tissue from WKY12 and SHR12, we focused on the same 453 well-resolved spots in a 12- to 90-kD molecular weight that we previously analyzed (6). This analysis showed significant differences in only 15 analyzed spots ($P < 0.05$). Accurate protein identification was achieved for 12 spots and failed in three. From these 12 identified protein spots, we excluded two spots that showed significant altered expression levels in the SHR12S group compared with normal heart, but the identification of these spots could be considered as plasma contaminant (albumin). The identification of the 10 remaining spots belongs to nine unique proteins, three of them common to other spots.

Oxidative Stress and Activation of NF-κB

We measured serum levels of 8-iso-PGF$_{2α}$, an oxidative stress marker. SHR48 showed significantly increased levels of 8-iso-PGF$_{2α}$ compared with WKY48 (10,591 ± 1892 versus 42,499 ± 8260 pg/ml; $P < 0.0001$), whereas rats that were treated with quinapril alone or in combination with doxazosin showed reduced levels compared with SHR48 but significantly higher than WKY48 (QUI 24,627 ± 2206 pg/ml; DXQ 24,260 ± 2515 pg/ml; $P < 0.008$ QUI and DXQ versus SHR48; $P < 0.02$ versus WKY48).

NF-κB activation was evaluated by EMSA in both heart and kidneys and by Southwestern in aorta. We showed that activated NF-κB was increased in all tissues in the SHR48 group compared with WKY48. Treated rats also showed similar NF-κB activation in both aorta and kidney than in the WKY48 group (WKY48 3.5 ± 0.4; QUI 3.5 ± 0.3; DXQ 3.4 ± 0.6; SHR48 4.9 ± 0.4; $P < 0.03$ all groups versus SHR48 in kidney; Figure 1A). Activated NF-κB in the heart also was reduced in treated groups compared with SHR48 but remained significantly increased versus WKY48 (Figure 1B).

Discussion

We observed 15 altered proteins in the early stage of LVH in the SHR12 group compared with their normotensive counterpart WKY rats (Tables 1 and 2). These proteins participate in several biologic processes, such as oxygen transport, electron transport system, glycolysis, stress, and contractility. Myoglobin levels are decreased in SHR12. Myoglobin is an intracellular hemoprotein that is expressed in the heart and oxidative skeletal myofibers and serves as a reserve supply of oxygen to facilitate the movement of oxygen within muscles (16). We also noted decreased levels of the small heat-shock protein β-6 (HSP-B6), a molecular chaperone that maintains both the morphologic and the functional integrity of the cardiomyocytes and increases tolerance against various types of stress as in adult rat cardiomyocytes. Furthermore, overexpression of HSP-20 protects rat cardiomyocytes from apoptosis that is triggered by activation of the cyclic adenosine monophosphate-protein kinase A pathway and is associated with inhibition of caspase-3 activity (17).

Surprising, other proteins that were downregulated in SHR12 versus WKY12 were the contractile protein tropomyosin 1 α chain and the glycolytic enzyme pyruvate dehydrogenase (lipoamide β). These findings oppose those of established LVH that showed an upregulation of tropomyosin 1 α (in other spots than in early-stage LVH model) and in two glycolytic enzymes (enolase-1 α and aldehyde dehydrogenase). These suggest a different regulation of these processes, depending on the stage of the disease.

Table 1. List of identified differentially expressed proteins in SHR left ventricular tissue: Spots that are altered exclusively in early LVH

<table>
<thead>
<tr>
<th>Spot No.</th>
<th>Protein Identified</th>
<th>Accession No.</th>
<th>Functional Class</th>
<th>Ratio Expression Level</th>
<th>p$^b$ WKY12 versus SHR12</th>
<th>Observed MW/pI</th>
<th>Theoretical MW/pI</th>
<th>Mowse$^d$ Score</th>
<th>Coverage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1114</td>
<td>Myoglobin</td>
<td>Q9QZ76</td>
<td>Oxygen transport</td>
<td>0.23 (1)</td>
<td>0.027</td>
<td>16.5/4.6</td>
<td>17.2/7.8</td>
<td>86</td>
<td>41</td>
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<tr>
<td>2104</td>
<td>Cytochrome c oxidase</td>
<td>P12075</td>
<td>Energy/metabolism</td>
<td>1.49 (1)</td>
<td>0.015</td>
<td>14.7/5.0</td>
<td>13.0/6.3</td>
<td>91</td>
<td>34</td>
</tr>
<tr>
<td>2803</td>
<td>Tropomyosin 1α chain</td>
<td>P04692</td>
<td>Cytoskeletal</td>
<td>0.88 (1)</td>
<td>0.013</td>
<td>33.0/5.1</td>
<td>32.7/4.7</td>
<td>294</td>
<td>56.7</td>
</tr>
<tr>
<td>4704</td>
<td>Pyruvate dehydrogenase (lipoamide β)</td>
<td>Q6YA95</td>
<td>Energy/metabolism</td>
<td>0.61 (1)</td>
<td>0.01</td>
<td>30.4/5.6</td>
<td>38.9/6.2</td>
<td>136</td>
<td>40</td>
</tr>
<tr>
<td>6301</td>
<td>Heat-shock protein β-6</td>
<td>P97541</td>
<td>Stress</td>
<td>0.37 (1)</td>
<td>0.017</td>
<td>18.1/6.2</td>
<td>17.6/6.1</td>
<td>122</td>
<td>44</td>
</tr>
<tr>
<td>6305</td>
<td>ATP synthase subunit d</td>
<td>P31399</td>
<td>Energy/metabolism</td>
<td>1.75 (1)</td>
<td>0.031</td>
<td>21.8/6.3</td>
<td>18.9/6.2</td>
<td>135</td>
<td>50</td>
</tr>
<tr>
<td>7403</td>
<td>DJ-1 protein</td>
<td>O88767</td>
<td>Stress</td>
<td>2.41 (1)</td>
<td>0.038</td>
<td>22.9/6.5</td>
<td>20.0/6.3</td>
<td>214</td>
<td>78</td>
</tr>
</tbody>
</table>

$^a$LVH, left ventricular hypertrophy; MW, molecular weight; pI, isoelectric point.

The t test was used for statistical comparison.

Theoretical pI and MW derived from NCBInr and SwissProt/TrEMBL databases.

Mowse score is the scored based on mowse algorithm when the protein identification was performed by peptide mass fingerprinting (matrix-assisted laser desorption ionization-mass spectrometry [MALDI-MS]) with the search program Mascot (www.matrixscience.com).
Table 2. List of identified differentially expressed proteins in SHR left ventricular tissue: Spots that are altered in early and late LVH

<table>
<thead>
<tr>
<th>Spot No.</th>
<th>Protein Identified</th>
<th>Accession No.</th>
<th>Functional Class</th>
<th>Ratio Expression Level</th>
<th>P WKY12 versus SHR12</th>
<th>Ratio Expression Level</th>
<th>P WKY48 versus SHR48</th>
<th>Observed MW/pI</th>
<th>Theoretical MW/pI</th>
<th>Mowse Score</th>
<th>Coverage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6704</td>
<td>Calsarcin-1</td>
<td>XP_215692</td>
<td>Signaling</td>
<td>1.88 (↑)</td>
<td>0.016</td>
<td>1.77 (↑)</td>
<td>0.031</td>
<td>29.9/6.3</td>
<td>29.8/7.0</td>
<td>87</td>
<td>25</td>
</tr>
<tr>
<td>7302</td>
<td>Ubiquinone biosynthesis protein COQ7 homolog</td>
<td>Q63619</td>
<td>Energy/metabolism</td>
<td>&lt;0.1 (↓)</td>
<td>0.006</td>
<td>&lt;0.1 (↓)</td>
<td>0.001</td>
<td>18.9/6.4</td>
<td>20.1/5.6</td>
<td>74</td>
<td>26</td>
</tr>
<tr>
<td>7808</td>
<td>Calsarcin-1</td>
<td>XP_215692</td>
<td>Signaling</td>
<td>2.27 (↑)</td>
<td>0.038</td>
<td>1.55 (↑)</td>
<td>0.048</td>
<td>29.9/6.7</td>
<td>29.8/7.0</td>
<td>78</td>
<td>29</td>
</tr>
</tbody>
</table>

*The t test was used for statistical comparison.

bTheoretical pI and MW derived from NCBInr and SwissProt/TrEML databases.

cMowse score is the score based on the mowse algorithm when the protein identification was performed by peptide mass fingerprinting (MALDI-MS) with the search program Mascot (www.matrixscience.com).

Figure 1. Activation of NF-κB transcription factor. (A) Representative images of the in situ detection of the NF-κB in the aorta sections of the various groups at 48 wk (Southwestern histochemistry): Normotensive control (WKY48; A), SHR without treatment (SHR48; B), SHR that received a combined therapy that consisted of 15 mg/kg per d doxazosin plus 1.6 mg/kg per d quinapril (C), and SHR that were treated with 16 mg/kg per d quinapril (D). (B) Representative electrophoretic mobility shift assay experiment from cardiac tissue and densitometric analysis of the results expressed as mean ± SEM of six to seven rats of each group. *P < 0.05 versus SHR48; †P < 0.05 versus WKY48. Magnification ×20.
We also observed increased DJ-1 protein expression levels in the SHR12 group, compared with normotensive control, which has chaperone function and has been described to protect neurons from cell death (18,19). We also observed upregulated in the heart of SHR12 versus WKY12 two proteins that belong to the mitochondrial electron transport system: Cytochrome c oxidase polypeptide Vb (mitochondrial precursor) and ATP synthase subunit d.

It is interesting that we found three altered spots that belong to two unique proteins that were equally expressed at both early and established LVH. Furthermore, these spots were not normalized in treated rats. These spots corresponded to ubiquinone biosynthesis protein COQ7 homolog (SSP 7302) and CS-1 (SSP 6704 and 7708). The former enzyme was >10-fold decreased in SRH12, SHR48, QUI, and DXQ compared with each respective control. Ubiquinone biosynthesis protein COQ7 homolog is a potential central metabolic regulator that is expressed dominantly in heart and skeletal muscle. It is involved in ubiquinone biosynthesis and, hence, required for respiration and gluconeogenesis.

Cardiac CS-1 was represented in two different spots, and both were upregulated in SHR12, SHR48, QUI, and DXQ groups compared with each respective control counterpart WKY rats. Calsarcins belong to a highly conserved protein family that interacts with calcineurin and the Z-disc proteins. In humans, they present two conserved domains at the carboxyl terminus, the calcineurin-binding region and α-actinin-binding region (8), that also are well conserved in other animals (20).

Because ubiquinone biosynthesis protein COQ7 homolog and CS-1 are common proteins in two different states of hypertension (early and late), these may be two potential targets to develop new antihypertrophy drugs in the setting of hypertension-induced LVH. CS-1 protein has an important role as a negative regulator of calcineurin signaling in striated muscle of hypertension. CS-1 protein has an important role as a targets to develop new antihypertrophy drugs in the setting of hypotension (early and late), these may be two potential and CS-1 are common proteins in two different states of animals (20).

We previously reported than SHR48 showed established LVH and severe cardiac injury, and their proteomic profile demonstrated decreased levels of antioxidant proteins and imbalance of many mitochondrial proteins, suggesting a marked oxidative stress in this setting (6). We checked the systemic oxidative stress showing that SHR48 presented significant increased levels of 8-iso-PGF$_2\alpha$ compared with WKY48 and that rats that were treated with both the combined therapy (low quinapril dosages plus doxazosin) and high quinapril dosages showed reduced levels compared with SHR48 but significantly higher than WKY48. The activation of NF-κB by ROS remained to a lesser extent in heart-treated rats but was not normalized as in the other tested organs (kidney or aorta). This could be due to the interaction of specific cardiac proteins with some components of the NF-κB pathway. In this sense, CS-1 could be a good candidate because it indirectly activates NF-κB through inactivation of IkBβ (21), expressed preferentially in heart, in a lesser manner in smooth muscle cells, but not in the kidneys. In our model, we noted an upregulation of the cardiac CS-1 coinciding with LVH. CS-1, which inhibits the NF-AT activation via calcineurin binding, could facilitate the activation of NF-κB via calcineurin inactivating IkBβ. Further investigations are necessary to elucidate the exact role of CS-1 and other proteins, such as ubiquinone biosynthesis protein COQ7, in the setting of hypertension-induced LVH.

Acknowledgments

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References

11. Higuchi M, Manna SK, Sasaki R, Aggarwal BB: Regulation of the activation of nuclear factor kappaB by mitochondrial
respiratory function: Evidence for the reactive oxygen species-dependent and -independent pathways. *Antioxid Redox Signal* 4: 945–955, 2002


