A Proteomic Approach to Determine Changes in Proteins Involved in the Myocardial Metabolism in Left Ventricles of Spontaneously Hypertensive Rats

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Key Words

Myocardial metabolism • Hypertension • Proteomics

Abstract

Background: Different works have suggested that in the hypertrophied heart the energy metabolic pathway shifts to glycolysis. Our aim was to evaluate using proteomics the expression of proteins associated with different energetic metabolic pathways in hypertrophied left ventricles of spontaneously hypertensive rats (SHR). **Methods:** 24-weeks-old SHR with stable hypertension and established left ventricle hypertrophy were used. Normotensive Wistar Kyoto rats were used as control. Proteins from left ventricles were analyzed by 2-dimensional electrophoresis and identified by comparison with a virtual rat heart proteomic map and mass spectrometry. **Results:** Enoyl-CoA hydratase expression, an enzyme involved in fatty acid β -oxidation, was reduced whereas the expression of other β -oxidation enzymes, 3-ketoacyl-CoA thiolase and the mitochondrial precursor of acyl-CoA thioester hydrolase, was increased in the hypertrophied left ventricles. The expression of two enzymes involved in the first steps of glycolysis, fructose biphosphate aldolase and triosephosphate

isomerase, was reduced in the left ventricle of SHR. Pyruvate dehydrogenase expression, enzyme involved in glucose oxidation, was enhanced in the hypertrophied ventricles whereas proteins of the tricarboxylic acid cycle were not modified. Proteins involved in the mitochondrial oxidative phosphorylation were overexpressed whereas the α -subunit of the mitochondrial precursor of ATP synthase was downexpressed. **Conclusions:** Several proteins involved in the main energy metabolic pathways were up and downexpressed. Moreover, our results seem to suggest that probably neither fatty acid β -oxidation nor glycolysis are the only sources for energy in the hypertrophied left ventricle.

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Introduction

Cardiac hypertrophy and more particularly left ventricular hypertrophy have been considered as a useful adaptive mechanism for the increased cardiac after load in patients with hypertension [1]. Although left ventricular hypertrophy is a leading cause of congestive heart failure, the transition to failure is not completely understood, in

part due to the lack of complete inventory of proteins associated with left ventricular hypertrophy. In this regard, most of the studies that evaluate changes in the expression of proteins in cardiac hypertrophy have been focused on the structural and contractile machinery, however, bioenergetic compensation must be also integrated within the mechanisms of cardiac adaptation [2]. In this regard, since the adult mammalian heart has high energy requirements, it is predictable that the pathophysiological derangements associated with cardiac hypertrophy may induce a series of adaptive and possibly maladaptive energy metabolic responses which may contribute to the detrimental functionality of the hypertrophied myocardium. In the heart, ATP is mainly synthesized in the mitochondria through oxidative phosphorylation and transported to the contractile apparatus, where it is consumed by myosin ATPase to generate force. In the non-hypertrophied hearts, the main energy substrate comes from the long-chain fatty acid β -oxidation. Several studies have suggested that oxidation of long-chain fatty acids is reduced in hypertrophied hearts and other ATP-generating sources must be consumed to maintain the ATP supply [3]. In this regard, in the hypertrophied heart the reduced fatty acid oxidation seems to be compensated by an increased contribution of glycolysis to ATP production, although, this remains controversial [4]. Therefore, it could be interesting to analyze, in an integrated form, changes in the expression of proteins involved in the main metabolic pathways once the ventricular hypertrophy stage is established.

An alternative approach in the analysis of protein expression patterns is the use of two-dimensional electrophoresis (2-DE) in combination with mass spectrometry which has been termed as proteomics. Proteomics is a powerful technique to measure changes in the expression of proteins and more importantly proteomics also allow us to identify changes in protein isotypes which it is not easily available by the most current technologies to determine proteins such as flow cytometry and Western blotting. This technology is now emerging in the study of complex pathophysiological disorders, including cardiovascular diseases. Therefore, it could be useful to determine by proteomics the *in vivo* modifications of the expression of proteins in hypertrophic left ventricles in a controlled animal model, such as spontaneously hypertensive rats (SHR). SHR provides a useful experimental model of pressure overload that develops hypertension gradually and has demonstrated many similarities with human essential hypertension, including the development of left ventricular hypertrophy

[5]. Thus, our aim was to determine using proteomics, the expression of proteins involved in the main energy metabolic pathways in left ventricles of normotensive and SHR.

Materials and Methods

Study design

The study was conducted in male 24 weeks-old SHR (n=9) and their normotensive controls, Wistar Kyoto rats (n=11). SHR have both hypertension and an established hypertrophy stage from 18 weeks of age. Animals were fed with a standard chow ad libitum (A=04; PanLab, Barcelona, Spain) and had free access to drink water. Systolic blood pressure (SBP) was measured by a tail-cuff plethysmograph (Narco Bio-Systems, Houston, TX), as previously described [6]. On the day of the experiment, the animals were killed by decapitation and the heart was carefully removed and weighed. Isolation and manipulation of hearts were always performed under sterile conditions. The left ventricles were immediately frozen at -80°C . All experimental procedures were approved by the Animal Care and Use Committee of Complutense University, in accordance with the guidelines for ethical care of experimental animals of the European Union.

Arterial and left ventricular hemodynamic parameters

On the day of the experiment, the rats were anaesthetized with ketamine/xylazine mixture (90 and 5 mg/kg, i. p.) and a pressure transducer (Science FT211B of 1.6F diameter) was introduced through the right carotid artery into the left ventricle. During this procedure arterial blood pressure and the left ventricular systolic (LVSP) and end-diastolic pressure (LVEDP) were measured. The catheter was connected to a data acquisition system (PowerLab/800, AD Instruments). Signals were monitored and digitally stored for their analysis with the commercial software Chart for Windows (v4.2). The maximum positive value of the first derivative of the pressure that occurs during the cardiac cycle ($+dP/dt$) and the maximum negative value of the first derivative of the pressure that occurs during the cardiac cycle ($-dP/dt$) together with heart rate were also determined and recorded (Mc Lab 8E, AD Instruments). $+dP/dt$ and $-dP/dt$ are isovolumic phase indices of left ventricular function.

The maintenance of diastolic blood pressure at proper values was the guarantee that the aortic valve was not damaged during the procedure. After arterial and left ventricular pressure recording, the animals were killed by decapitation and the heart was carefully removed. Isolation and manipulation of hearts were always performed under sterile conditions. Heart weight was normalized to body weight ($\text{mg} \times \text{g}^{-1}$). This ratio was used as an index of heart hypertrophy.

Two dimensional electrophoresis (2-DE)

After the hemodynamic study was performed, the 20 left ventricles were isolated and homogenized in a buffer contain-

Fig. 1. Left: Representative 2-DE map of a left ventricle from SHR using a pH range from 3 to 10. Several areas were analyzed (B to S) in each group of rats (9 SHR and 11 normotensive Wistar Kyoto rats). Right: Representative 2-DE images of the expression of proteins in which significant statistical differences were found. α -actin was identified in area A and was used to normalize total spot volume in each of the other areas.

ing 8 mol/L urea, 2% CHAPS w/v, 40 mmol/L dithiothreitol, 0.2% Bio-Lyte™ ampholyte (Bio-Rad) and 0.01% w/v bromophenol blue. The samples (250 µg protein / sample) were loaded on immobilized gradient IPG strips (pH 3-10) and isoelectric focusing was performed using a Protean IEF cell system (Bio-Rad). The gels were actively rehydrated at 50V for 60 hours, then rapid and linear voltage ramping steps, limited by a maximum current of 50 µA per gel [7]. In the second dimension, the proteins from the strips were resolved on 10% SDS-PAGE gels using a Protean II XL system (Bio-Rad). Afterwards, the gels were fixed and stained using Plus One Silver Staining kit (GE Healthcare). In brief, the gels were fixed in a solution containing ethanol and glacial acetic acid in distilled water for 60 minutes. Afterwards, the gels were treated in a sensitizing solution containing ethanol, sodium thiosulfate, and sodium acetate in distilled water during 60 minutes. Briefly, the gels were washed four times in distilled water for 15 minutes each time. Afterwards, the washed gels were stained in a silver solution containing silver nitrate solution and distilled water during 1 hour as manufacturers recommended. Then, the gels were washed twice in distilled water and treated in developing solution containing sodium carbonate, formaldehyde and distilled water for approximately 2-4 minutes and stopped with stopping solution containing EDTA- Na_2 2 H₂O and distilled water during 1 hour. The stained gels were then washed twice in distilled water for 5 minutes each time.

Image acquisition and analysis

The silver-stained gels were scanned in a UMAX POWERLOOK III Scanner operated by the software Magic Scan V 4.5. Intensity calibration was carried out using an intensity stepwedge prior to gel image capture. Image analysis was

carried out using PD Quest 6.2.1 and Quantity One 4.2.3 (Bio-Rad). Each spot intensity volume was processed by background subtraction and total spot volume normalized to the corresponding densitometric value of cardiac α -actin (Fig. 1, area A). The densitometric values of cardiac α -actin in the left ventricles from WKY and SHR were not different (WKY: 1728.15 ± 338.4 , SHR: 982.77 ± 161.6 , $p = \text{NS}$). The identity of each spot was performed by comparison with the rat heart proteomic map published by Li et al [8]. For calculations, two gels were developed for each ventricle and the mean of the two densitometric values for each spot was used for the statistical analysis.

Mass spectrometry

Some spots were manually excised from the gels using biopsy punches. Spots from three different gels were obtained and analyzed. The silver staining was then solubilized and removed by a solution contained sodium thiosphate 100 mmol/L and potassium ferricyanide 30 mmol/L, and the spots were washed three times with milliQ water. The samples were shrunk with 100% acetonitrile, and dried in a SpeedVac. The samples were digested overnight at 37°C with 15 ng/µL sequencing grade trypsin (Promega) in 25 mmol/L ammonium bicarbonate (pH 8.5) [7]. After digestion, the peptides were extracted with 100 mmol/L ammonium bicarbonate and then, the extracts were lyophilized and resuspended in 0.1% TFA. The peptides were purified by use of a mmol/l-18 Zip tips (Millipore). For MS analysis, 1 µL of purified extracts was mixed with a 1 µL of α -cyano 4-hydroxy-transcinnamic matrix (Sigma) in 50% acetonitrile and 1 µL of this mixture was spotted on to a Mald plate and allowed to air-dry at room temperature. Mass spectrometry (MS) analyses were performed in a 4700 Proteomic Analyzer (Applied Biosystem) and operated in a reflector posi-

Table 1. Physiological parameters of the animals. SBP: systolic blood pressure (mmHg); DBP: diastolic blood pressure (mmHg); HR: heart rate, expressed as beats per minute (bpm); HRW: heart relative weight, expressed as g of heart / 100g of body weight (g/100 g bw); LVSP: left ventricular systolic pressure (mmHg). LVEDP: left ventricular end-diastolic pressure (mmHg). dP/dt_{max} : positive first derivate of pressure with respect to time (mmHg/s). dP/dt_{min} : negative first derivate of pressure with respect to time (mmHg/s). * $p < 0.05$ with respect to left ventricle from WKY rats.

	WKY rats	SHR rats	p-value
SBP	117 ± 5.89	133 ± 7.49*	0.0001
DBP	80 ± 4.69	95 ± 5.95*	0.0001
HR	237 ± 3.77	231 ± 7.42	0.4607
HRW	0.28 ± 0.003	0.35 ± 0.009*	0.0001
LVSP	122 ± 3.41	135 ± 3.83*	0.0227
LVEDP	3.5 ± 0.165	5 ± 0.294*	0.0003
dP/dt_{max}	6807 ± 116.08	8875 ± 370.36*	0.0001
dP/dt_{min}	-5582 ± 181.8	-5894 ± 135.1	0.1639

tive mode. All mass spectra were calibrated using a standard peptide mixture (Applied Biosystem). The analysis in a MS mode produced peptide mass fingerprints, and some peptides observed were further analyzed in a MS/MS mode, the criteria used for protein identification were 50 ppm and carbamidomethyl modification. Peptides with a signal-to-noise greater than 20 were considered in the Mascot Database for protein identification. For protein identification, Mascot database 1.9 (<http://www.matrixscience.com>) was used as algorithm to match the peptides obtained by mass spectrometry.

Western blot analysis

The protein expression of triosephosphate isomerase, fructose biphosphate aldolase, malate dehydrogenase, NADH dehydrogenase and mitochondrial ATP synthase α -chain were analyzed by Western blot. In brief, homogenized left ventricle cells were solubilized in Laemmli buffer containing 2-mercaptoethanol. The proteins obtained were separated on denaturing SDS/15% (w/v) polyacrylamide gels. Equal amount of proteins (20 μ g/lane), estimated by bicinchoninic acid reagent (Pierce), were loaded. Proteins were then blotted onto nitrocellulose (Immobilion-P; Millipore) and the blots were blocked overnight at 4°C with 5% (w/v) non fat dry milk in TBS-T [20 mmol/L Tris/HCl (pH 5.2), 137 mmol/L NaCl and 0.1% Tween 20], as reported [9]. The membranes were incubated with monoclonal antibodies against either triosephosphate isomerase (1:500; sc-30145, Santa Cruz Biotechnology), fructose biphosphate aldolase (1:500; Aldolase A (N-15): sc-12059, Santa Cruz Biotechnology), malate dehydrogenase (1:1000; MDHC (N-18): sc-49234, Santa Cruz Biotechnology), NADH dehydrogenase (1:500; ND1 (C-18): sc-20493, Santa Cruz Biotechnology) and mitochondrial ATP synthase α chain (1:2000; ATP5A (15H4): sc-58613, Santa Cruz Biotechnology). After they were incubated with HRP (horseradish peroxidase)-conjugated anti-(mouse or goat IgG) antibodies at a dilution of 1:2000. The proteins were then detected by enhanced chemiluminescence (ECL®, Amersham Biosciences) and evaluated by densitometry (Quantity One; Bio-Rad Laboratories). Pre-stained protein markers (Sigma) were used for molecular mass determinations. To compare the protein expression of the different proteins with the expression of another constitutive protein, the expression of β -actin was also analyzed. For this purpose, a parallel gel with identical samples was run and, after blotting onto nitrocellulose, Western blot

analysis was performed with an anti- β -actin monoclonal antibody (1:2000, Sigma-Aldrich, Saint Louis, USA).

Detection of pyruvate

The amount of pyruvate present in the left ventricles was quantified using a Pyruvate assay Kit (BioVision, USA). For this purpose, 80 μ g of each homogenized left ventricle was used to assay pyruvate concentration using the colorimetric assay following the manufacturer's instructions.

Statistical analysis

Results are expressed as mean $\pm$ SEM. To determine statistical differences, Mann-Whitney's test was used in the SPSS 12.0 program (SPSS Inc.). A p value < 0.05 was considered statistically significant.

Results

Heart relative weight and cardiac hemodynamics

As expected, SHR presented higher ($p < 0.05$) SBP and DBP levels than WKY (Table 1). However, heart rate was similar in both hypertensive and normotensive animals (Table 1). No differences in body weight were observed between both groups. However, SHR had a higher ($p < 0.05$) heart relative weight than WKY (Table 1). Both left ventricular end diastolic pressure (LVEDP) and left ventricular maximal systolic pressure (LVSP) were higher ($p < 0.05$) in SHR than in WKY (Table 1). These changes in SHR were associated with increases in $+dP/dt$ and no changes in $-dP/dt$ (Table 1).

Analysis of proteins

More than 248 spots were detected in two-dimensional gel electrophoresis of ventricles from normotensive rats ranging from 15 to 200 kDa with a pH from 3 to 10. In two-dimensional gels of ventricles from SHR around 340 spots were detected ($p < 0.05$ with respect to normotensive hearts). It may be associated with

Protein	Theoretical Mass (kDa/pI)	Experimental Mass (kDa/pI)	DATABASE accession number	Peptides Matched	Confirmation Method	Sequence Coverage (MS)
Actin, alpha cardiac	42.3/5.23	42.3/5.23	P68035	9 CDDEETALVCDNGSGLVK / AGFAGDDAPR / AVFPSIVGRP / VAPEEHPIILLTEAPLNPK / TTGIVLDSGDGVTHNVPIYEGYALPHAIMR / GYSFVTTAER / SYELPDGQVITIGNER / DLYANNVLSGGTTMYPGIADR / QEYEDEAGPSIVHR	MS	39%
Enoyl-CoA hydratase	28.2/8.1	27.9/8.00	P14604	-	Heart map	-
3-ketoacyl-CoA thiolase	42.2/8.09	42.9/7.26	P13437	2 VGVPITETGALTNR / YALQSQQR	MS/MS	5%
Acyl CoA thioester hydrolase mitochondrial precursor	49.9/7.68	42.8/7.01	O55171	1 GLAPEQPVTLR	MS/MS	2%
Fructose bisphosphate aldolase	39.3/9.0	43.1/9.20	P05065	-	Heart map	-
Triosephosphate isomerase	27.3/6.51	24.7/7.81	P48500	6 LPADTEVVCAPPTAYIDFAR / DLGATWVVLGHSE / HIFGESDELIGQK / VVFEQTK / TATPQQAQEVHEK / CNVSEGVACQTRIIYGGSVTGATCK	MS MS/MS	37%
Glyceraldehyde-3 phosphate dehydrogenase	35.9/8.44	33.4/8.65	P04797	5 VGVNGFGR / LVINGKPITIFQE / LTGMAFR / VPIPNVSVVDLTC / LISWYDNEYGYSN	MS	17%
α/β -enolase isotype 1	47.1/7.74	45.2/6.99	P04764/ P15429	5 AAVPSGASTGIYEALCLR / KLSVVDQEK / VVIGMDVAASEFYR / TGAPCRSER / IEEALGDKAVFAGR	MS MS/MS	14%
α/β -enolase isotype 2	47.1/7.74	45.4/7.06	P04764 / P15429	3 AAVPSGASTGIYEALCLR / VVIGMDVAASEFYR / LAQSNWGVVMVSHR	MS MS/MS	10%
Pyruvate dehydrogenase	38.8/5.9	34.1/5.80	50925725	-	Jin X et al [11]	-
Isocitrate dehydrogenase	39.6/6.5	37.1/6.72	16758446	-	Jin X et al [11]	-
Malate DH mitochondrial isotype 1	36.1/8.9	33.7/8.80	P04636	7 GYLGPEQLPDCLK / CDVVVIPAGVPR / IFGVTTLDIVR / TIPLISQCTPK / AGAGSATLSMAYA / VFLSDVAMNGK / ETECTYFSTPLLL	MS	27%
Malate DH mitochondrial isotype 2	33.8/9.2	33.8/9.20	P04636	-	Heart map	-
Malate DH cytoplasmic isotype 1	36.5/6.16	33.5/5.89	P14152	4 SQGTALEK / ENFSCLTR / GEFITTVQQR / FVEGLPINDFSR	MS MS/MS	11%
Malate DH cytoplasmic isotype 2	36.5/6.16	33.3/6.15	P14152	2 GEFITTVQQR / FVEGLPINDFSR	MS/MS	6%
Ubiquinol-cytochrome c reductase	29.7/9.04	26.2/7.79	P20788	1 VPDFEDYR	MS/MS	2%
NADH dehydrogenase	31.9/5.4	39.3/6.85	46391108	-	Jin X et al [11]	-
Electron transfer flavoprotein α -subunit mitochondrial precursor	35.2/8.67	31.2/7.10	P13803	6 VLVAQHDAVK / SPDTFVR / GTSFEAAAASGGG / APSSSSAGISEWLDQK / SDRPELTGAK / AADAGFVPNDMQVGGQTK	MS	24%
Electron transfer flavoprotein β -subunit mitochondrial precursor	27.5/8.57	27.5/8.57	Q9DCW4	4 HSMNPFCEIAVEEAVR / TALAMGADR / EIDGGLETLRLKLPVVTADLR / VSVISVEEPPQR	MS MS/MS	23%
ATP synthase alpha chain mitochondrial precursor	22.2/6.2	22.2/6.7	P15999 / PWB0A	8 LSVRVAAAVAR / ILGADTSVDLEETGGR / EAYPGDVFLHSR / GIDRPAINVGLSVSR / VGSAAQTR / LELAQR / GYLDKLEPSK / EIVTNFLAGFEA	MS MS/MS	16%
ATP synthase beta chain mitochondrial precursor	50.7/4.9	50.7/4.9	A28701	16 LVLEVAQHLGESTVR / TIAMDGTEGLVR / VLDSGAPIKIPVGPETLGR / IMNVIGEPIDER / VVDLAPYAK / IGLFGGAGVK / AHGGYSVFAGVGER / EGNLDYHEMISGVINLK / VALVYQGMNEPPGA R / VALTGLTVAEYFR / FTQAGSEVSALLGR / IPSAVGYQPTLATDMGMTQER / AIAELGHYPVDPPLDSTSR / IMDPNIVGSEHYDVAR / ILQDYK / ADKLAEHGS	MS	47%
Creatine kinase M chain	43.2/6.58	41.3/7.26	P00564	4 VLTPDLYNKL / DLFDPIQDR / FEEILTR / GTGGVDTAAVGAFFDYSNADR	MS	12%

Table 2. Methods followed to identify the left ventricle proteins after 2D electrophoresis. MS: mass spectrometry; MALDI-TOF MS analyses; Heart map: <http://www.mpiib-berlin.mpg.de/2D-PAGE/RAT-HEART/2d/>; MS/MS: Tandem mass spectrometry. MALDI-TOF MS= matrix-assisted laser desorption/ionization time of flight mass spectrometry. Jin X et al, [11].

Protein	WKY rats	SHR rats	p-value
Enoyl-CoA hydratase	9.17 ± 2.4	0.90 ± 0.3*	0.012
3-ketoacyl-CoA thiolase	1.51 ± 0.4	4.58 ± 1.7*	0.033
Acyl CoA thioester hydrolase mitochondrial precursor	1.41 ± 0.4	6.42 ± 1.3*	0.0009
Fructose biphosphate aldolase	2.76 ± 0.7	0.80 ± 0.4*	0.011
Triosephosphate isomerase	7.78 ± 1.2	4.54 ± 1.3*	0.05
Glyceraldehyde-3 phosphate dehydrogenase	30.04 ± 5.3	30.66 ± 8.4	1
α/β -enolase isotype 1	3.63 ± 0.6	8.36 ± 1.5*	0.012
α/β -enolase isotype 2	2.04 ± 0.7	4.48 ± 1.1*	0.025

Table 3. Densitometric analysis in arbitrary units of the expression of proteins associated with fatty acid β -oxidation and glycolysis in the 2-DE gels of left ventricles. Densitometric results are represented as mean $\pm$ SEM. * $p < 0.05$ with respect to left ventricle from WKY rats.

Table 4. Densitometric analysis in arbitrary units of the proteins associated with pyruvate dehydrogenase and tri-carboxylic acid cycle in the 2-DE gels of left ventricles. Densitometric results are represented as mean $\pm$ SEM. * $p < 0.05$ with respect to left ventricle from WKY rats.

Protein	WKY rats	SHR rats	p-value
Isocitrate dehydrogenase	1.20 ± 0.2	2.33 ± 0.7	0.395
Malate dehydrogenase mitochondrial isotype 1	19.72 ± 4.4	19.35 ± 3.9	1
Malate dehydrogenase mitochondrial isotype 2	15.08 ± 3.2	15.02 ± 5.8	0.656
Malate dehydrogenase cytoplasmic isotype 1	5.37 ± 1.3	5.10 ± 1.1	0.882
Malate dehydrogenase cytoplasmic isotype 2	6.73 ± 1.4	10.72 ± 2.6	0.201
Pyruvate dehydrogenase	2.01 ± 0.2	4.40 ± 0.8*	0.009

the well demonstrated increased global protein synthesis, particularly those associated with cell growth and cellular matrix in the hypertrophied stage (for review see [10]).

In most cases, the identity of the measured spots was performed using the rat heart map published by Li et al [8] and in some cases it was further confirmed by mass spectrometry (Table 2). For this purpose, the spots were subjected to MALDI-TOF mass spectrometry and database searching (<http://www.matrixscience.com>).

Proteins associated with fatty acid beta-oxidation

Three enzymes involved in the fatty acid β -oxidation cycle were identified: enoyl-CoA hydratase, 3-ketoacyl CoA thiolase and the mitochondrial precursor of acyl-CoA thioester hydrolase (Fig. 1 area B, C and D). Protein expression of both 3-ketoacyl CoA thiolase and the mitochondrial precursor of acyl-CoA thioester hydrolase in the 2-DE gels were significantly increased in left ventricles from SHR with respect to those from WKY rats (Table 3, Fig. 1). However, in the hypertrophied left ventricles the expression of enoyl-CoA hydratase was significantly reduced (Table 3, Fig. 1).

Proteins associated with glycolysis

Four cytosolic enzymes associated with glycolysis: fructose biphosphate aldolase, triosephosphate isomerase, two α/β -enolase isotypes and glyceraldehyde 3-phosphate dehydrogenase, were identified (Fig. 1, areas E, F, G and H). As shown in Table 3 and Fig. 1, the expression of both fructose biphosphate aldolase and triosephosphate isomerase in the 2-DE gels were significantly reduced in left ventricles from SHR with respect to those from WKY rats. The changes in the expression of triosephosphate isomerase and fructose biphosphate aldolase were further assessed by Western blot. As shown in Fig. 2, Western blot analysis demonstrated that the expression of both triosephosphate isomerase and fructose biphosphate aldolase were decreased in left ventricles of SHR with respect to those found in normotensive WKY rats.

The proteome map showed that the protein expression of glyceraldehyde 3-phosphate dehydrogenase was not modified in the hypertrophied left ventricles, whereas, the protein expression of two α/β -enolase isotypes were significantly increased (Table 3, Fig. 1).

Fig. 2. Left: Representative Western blot showing the content of triosephosphate isomerase (TPI), fructose bisphosphate aldolase (Aldolase A), malate dehydrogenase (malate DH), NADH dehydrogenase (NADH DH) and mitochondrial ATP synthase α chain (ATP5A) in left ventricles from SHR (n=9) and WKY (control group, n=11) rats. The expression of a constitutive protein, β -actin, is also shown. Right: Bar graphs shown the densitometric analysis (arbitrary units) of the Western blots normalized by the corresponding β -actin. Densitometric values are represented as mean $\pm$ SEM. * $p < 0.05$ with respect to the control group.

Pyruvate dehydrogenase and tricarboxylic acid cycle-associated proteins

The protein expression of pyruvate dehydrogenase, an enzyme included in the pyruvate dehydrogenase complex, was significantly enhanced in the 2-DE gels of the left ventricles of SHR with respect to those from WKY (Fig. 1, area I and Table 4). The pyruvate content in the left ventricles was also determined. As Fig. 3 shown, the hypertrophied left ventricles have a significative higher pyruvate content that those from WKY.

Two enzymes included in the tricarboxylic acid cycle, isocitrate dehydrogenase, and four malate dehydrogenase isotypes were also identified (Fig. 1 areas J, K and L). The expression of all of them was not modified in the 2-DE gels of the left ventricles from SHR with respect to WKY rats (Table 4, Fig. 1).

The malate dehydrogenase expression was further assessed by Western blot. As Fig. 2 shown, Western blot analysis demonstrated that the expression of malate dehydrogenase was not modified in the left ventricles of SHR with respect to those found in normotensive WKY rats.

Expression of proteins involved in mitochondrial oxidative phosphorylation

Three proteins involved in mitochondrial oxidative phosphorylation, NADH dehydrogenase, ubiquinol-cytochrome C reductase and mitochondrial precursor of α and β subunits of flavoprotein were identified (Fig. 1, areas M, N, O and P). The left ventricle expression of both ubiquinol-cytochrome C reductase and NADH dehydrogenase was increased in the 2-DE gels of the

Fig. 3. Pyruvate content in the left ventricle of SHR (n=9) and WKY (n=11) rats. Results are represented as mean $\pm$ SEM. * $p < 0.05$ with respect to WKY rats.

left ventricles from SHR (Table 5, Fig. 1). The protein expressions of mitochondrial precursor of the α and β subunits of the electron transfer flavoprotein were not different when the left ventricles of SHR and WKY rats were compared (Table 5, Fig. 1).

The change in the expression of NADH dehydrogenase was further assessed by Western blot. As shown in Fig. 2, Western blot analysis demonstrated that the expression of NADH dehydrogenase was increased in the left ventricles of SHR with respect to those found in normotensive WKY rats.

In areas Q and R (Fig. 1), the α and β subunits of the mitochondrial precursor of ATP synthase were detected. As shown in Table 5 and Fig. 1, the densitometric analysis of the 2-DE gels showed a significant increase in the expression of the β -subunit of the mitochondrial

Protein	WKY rats	SHR rats	p-value
Ubiquinol-cytochrome c reductase	5.35 ± 0.6	11.15 ± 2.5*	0.01
NADH dehydrogenase	2.95 ± 0.7	6.22 ± 1.4*	0.033
Electron transfer flavoprotein α -subunit mitochondrial precursor	9.36 ± 1.3	9.10 ± 0.9	0.882
Electron transfer flavoprotein β -subunit mitochondrial precursor	3.85 ± 0.8	2.84 ± 0.5	0.436
Mitochondrial precursor ATP synthase alpha chain	32.38 ± 4.9	19.17 ± 4.2*	0.031
Mitochondrial precursor ATP synthase beta chain	43.89 ± 5.2	83.15 ± 14.1*	0.001

Table 5. Densitometric analysis in arbitrary units of the expression of proteins associated with mitochondrial oxidative phosphorylation in the 2-DE gels of left ventricles. Densitometric results are represented as mean $\pm$ SEM. * $p < 0.05$ with respect to left ventricle from WKY rats.

precursor of ATP synthase. However, the expression of the α -subunit of the mitochondrial precursor of ATP synthase was found significantly reduced in the hypertrophied left ventricles (Table 5, Fig. 1). The reduction in the ATP synthase α -subunit expression in the hypertrophied left ventricles was also observed in Western blot experiments (Fig. 2).

Creatine kinase

Another protein identified in the left ventricles was creatine kinase M chain, (Fig. 1, area S). The expression of creatine kinase M chain was significantly enhanced in the 2-DE gels of the left ventricles from SHR with respect to those from WKY rats (20.39 ± 3.7 vs 8.96 ± 1.49 ; $p < 0.05$).

Discussion

The present study shows changes in the expression of proteins involved in different energetic metabolic pathways in hypertrophied left ventricles of SHR. We observed modifications in the expression of proteins involved in the fatty acid β -oxidation, glycolysis and mitochondrial oxidative phosphorylation.

In our knowledge, this is the first study that using proteomics was exclusively focused in the analysis of proteins involved in the main energetic metabolic pathways in the left hypertrophied ventricle of SHR. There are two previous proteomic studies in which proteins involved in the energetic metabolism of the hypertrophied ventricle had been partially analyzed. The first of them was performed using a smaller number of SHR ($n = 3$) than we here did and, in the second one, performed by Zhou et al, the number of the analyzed energetic metabolic-related proteins and protein isotypes for each metabolic pathway was markedly lesser than those determined in the present study which can lead to confusing conclusions

[11, 12].

Cardiac hypertrophy and hemodynamics

The development of hypertensive left ventricular hypertrophy is an adaptive response to pressure overload. SHR have been frequently used as an animal model of genetic hypertension which develops heart failure with aging, similarly to man. The results from the present study support previous findings showing cardiac hypertrophy in adult SHR accompanied by elevations of LVSP and $+dP/dt$, which should be considered as an adaptive compensating mechanism to an elevated after load. These results are in agreement with previous hemodynamic measurements showing increased systolic performance of SHR hearts [13, 14]. Also supporting previous observations, the present results show LVEDP elevation in SHR rats, indicating the existence of diastolic dysfunction, which is a common finding in hypertrophied hearts from SHR [15]. Diastolic dysfunction occurs when the ventricular chamber is unable to accept an adequate volume of blood during diastole at volumes sufficient to maintain an appropriate stroke volume [16, 17]. These abnormalities are caused by a decrease in ventricular relaxation and/or an increase in ventricular stiffness, which seems to be the case under the present conditions since $-dP/dt$ was not altered in the SHR [14].

It has been proposed that cardiac hypertrophy causes a breakdown in the energy metabolic system balance of heart. Several studies have shown a shift from the use of fatty acids towards the use of glucose as energy substrates [18]. Iemitsu et al. showed that the mRNA expression of key enzymes in the glycolytic metabolic pathway was markedly increased in the SHR hearts [19]. As it will be discussed later, our study showed moderate changes in fatty acid oxidation, glycolysis and TCA cycle, suggesting that the present conditions of diastolic dysfunction are characterized by a no marked shift towards the use of the glycolytic pathway. Moreover, the present results show

an important adaptation of mitochondrial oxidative phosphorylation to maintain a constant demand of ATP, as manifest by increased LVSP and +dP/dt.

Fatty acid β -oxidation associated proteins

Fatty acid β -oxidation, which converts fatty acids into acetyl-CoA, is the principal source of energy supply in non-hypertrophied hearts [20]. Previous studies have suggested the utility of the energy substrate shift from fatty acid oxidation to glycolysis in the hypertrophied heart [3]. In this regard, de las Fuentes et al have observed using positron tomography with 1-carbon-11 palmitate a decrease of myocardial long-chain fatty acid oxidation in the left ventricle of hypertensive patients which it was even associated with a reduction in myocardial efficiency [21]. In the present study, we have analyzed the expression of three proteins involved in fatty acid oxidation, enoyl-CoA hydratase, 3-ketoacyl CoA thiolase and the mitochondrial precursor of the acyl-CoA thioester hydrolase. Enoyl-CoA hydratase, an enzyme involved in the second step of the fatty acid-oxidation cycle that catabolyzes the formation of 3-hydroxyacyl-CoA, was found downexpressed in the hypertrophied left ventricles. However, the protein expression of 3-ketoacyl CoA thiolase, enzyme that promotes the reaction of β -ketoacyl-CoA to form acetyl CoA, was increased in the left ventricles of SHR. A number of investigators have reported a reduction in long-chain fatty acid β -oxidation mainly associated with lower levels of myocardial carnitine content in hypertrophied hearts [5]. Carnitine is absolutely essential for fatty acid translocation across the mitochondrial inner membrane to the site of β -oxidation [22]. However, our study suggests that the reported reduction of β -oxidation of long-chain fatty acids in the hypertrophied heart may be not only consequence of the reduction in the myocardial carnitine but also related to downexpression of one of the enzymes involved in the fatty acid β -oxidation cycle, the enoyl-CoA hydratase. In the same line of evidence, downexpression of mRNA levels encoding enzymes that catalyze the first and third steps of the fatty acid β -oxidation cycle (acyl-CoA dehydrogenase and β -hydroxyacyl CoA dehydrogenase) has been also reported in hypertrophied hearts [23].

Therefore, the β -oxidation process may be compromise by the observed downexpression of enoyl-CoA hydratase. Then, the depressed β -oxidation may result in excessive accumulation of fatty acid metabolites in the hypertrophied ventricle upstream to enoyl-CoA hydratase which may cause feedback inhibition of the β -oxidation pathway [24]. Therefore, as compensatory response to

attempt to reduce such fatty acid metabolites accumulation and to increase β -oxidation both 3-ketoacyl CoA thiolase, the acyl-CoA thioester hydrolase mitochondrial precursor may result overexpressed. In this regard, acyl-CoA thioester hydrolase hydrolyzes fatty acyl-CoA to free fatty acid and CoA-SH and it has been involved in the reduction of free fatty acid accumulation in heart mitochondrias from diabetic rats [25].

Metabolic enzymes associated with glycolysis

As a result of reduced fatty acid oxidation, an increased consumption of other substrates, mainly glucose, has been reported in hypertrophied hearts [26]. It has been mainly supported by the fact that glucose uptake seem to be increased in hypertrophied and mildly failing hearts, which has suggested an increased glycolysis [4, 27]. However, in our study, the expression of both fructose biphosphate aldolase and triosephosphate isomerase, was significantly reduced in the left ventricles from SHR. This finding was further confirmed by Western blot experiments in which the expression of triosephosphate isomerase was found reduced in the hypertrophied left ventricles. Glyceraldehyde-3-phosphate dehydrogenase and two α/β -enolase isotypes were also determined. Whereas the glyceraldehyde-3-phosphate dehydrogenase expression was not modified, the two α/β -enolase isotypes, which convert 2-phosphoglycerate into phosphoenolpyruvate, were upexpressed. As occurred for the fatty acid β -oxidation, the observed α/β -enolase upexpression could be related to a compensatory mechanism trying to increase glucose degradation. In this regard, Zhou et al in their proteomic study also found upregulated the expression of enolase in the hypertrophied heart. Since in this work there was only determined enolase within the glycolytic pathway the authors assumed a stimulated glycolysis in the hypertrophied ventricle [12]. However, since both fructose biphosphate aldolase and triosephosphate isomerase are key enzymes in the first steps of glycolysis, our results could not support an increased glycolysis in hypertrophied left ventricles. Moreover, the changes in the expression of the above mentioned glycolytic enzymes seem to be specific since the expression of other enzymes involved in glycolysis such as glyceraldehyde-3-phosphate dehydrogenase was not modified in the left ventricles of SHR.

The reported increased glucose uptake and the reduction in the protein expression of both fructose biphosphate aldolase and triosephosphate isomerase may favour the accumulation of metabolites upstream of these enzymes such as glucose-6-phosphate. In this regard, in

addition to being catabolyzed through glycolysis, glucose-6-phosphate is also the first substrate for glycogen production. Accordingly, cardiac glycogen accumulation in patients with left ventricle hypertrophy has been recently reported [28]. Moreover, it should be reminded that, although most of the consumed glucose is catabolyzed via glycolysis to pyruvate and then through the oxidative decarboxilation from pyruvate to acetyl-CoA, there are other catabolic glucose pathways that constitute the secondary metabolism of glucose. One of the main pathways of such secondary glucose metabolism is the pentose phosphate cycle. Zimmer et al have reported, using different models of cardiac hypertrophy in rats, that the activity of glucose-6-phosphate dehydrogenase and the available pool of 5-phosphoribosyl-1-pyrophosphate, which is synthesized from ribose-5-phosphate derived from the pentose phosphate pathway, were increased in the hypertrophied hearts, which it may suggest stimulation of this cycle in the cardiac hypertrophy [29]. Unfortunately, we could not identify in the proteomes any enzyme involved in the pentose phosphate cycle.

Glucose oxidation

Pyruvate is the end product of glycolysis. The pyruvate dehydrogenase complex, which is located in the mitochondria, mediates the oxidation of pyruvate to yield acetyl-CoA. Indeed, the pyruvate dehydrogenase complex is rate limiting for glucose oxidation in the heart. In the hypertrophied left ventricle, we have observed an increased expression of pyruvate dehydrogenase, one of the three enzymes included in the pyruvate dehydrogenase complex. In this regard, an increased pyruvate content was observed in the hypertrophied left ventricle which it may favour pyruvate dehydrogenase upexpression. Paradoxically, in our study while downexpression of two rate-limiting enzymes of glycolysis (fructose biphosphate aldolase and triosephosphate isomerase) was found in the hypertrophied left ventricles, an increased content of pyruvate (the end product of glycolysis) was also found in the same ventricles compared with those from WKY rats. However, it is important to remind that pyruvate may be also formed as intermediate metabolite in the aminoacid degradation of acetyl-CoA.

Tricarboxylic acid cycle

The common end-product of both fatty acid β -oxidation and glucose oxidation is acetyl-CoA. Acetyl-CoA is metabolized throughout the tricarboxylic acid cycle in the mitochondria. Our proteomic study showed that

the expression of two enzymes included in the tricarboxylic acid cycle, isocitrate dehydrogenase and four malate dehydrogenase isotypes, two mitochondrial and two cytoplasmic, were not modified in the left ventricle from SHR compared with those from normotensive WKY rats, suggesting that the tricarboxylic acid cycle is maintained in left ventricular hypertrophy. Accordingly, Garg et al failed to find modifications in the mRNA expression of the enzymes involved in the tricarboxylic acid cycle in cardiac hypertrophy associated with Chagas' disease [30].

Mitochondrial oxidative phosphorylation

Complex I (NADH dehydrogenase, flavoprotein) and complex II (ubiquinol-cytochrome C reductase) are parts of the electron transport chain for the generation of ATP in the mitochondria. Our study showed that the protein expression of both ubiquinol-cytochrome C reductase and NADH dehydrogenase were increased in the left ventricles of SHR compared with those from WKY rats. This finding may reflect an increased level of energy demand by the hypertrophied myocardium. Accordingly, Jin et al have also observed overexpression of ubiquinol-cytochrome c reductase and NADH-ubiquinone oxidoreductase (an enzyme included in complex I) in hypertrophied left ventricles of SHR [11].

High rates of myocardial energy production are required to maintain the constant demand of the working heart for ATP. However, a general decrease of ATP supply has been demonstrated in the hypertrophied left ventricles of SHR, which may be apparently in contrast with our observation of an overexpression of ubiquinol-cytochrome c reductase and NADH dehydrogenase. However, we have also identified in the 2-DE gels the α and β subunits of the mitochondrial precursor of ATP synthase. The mitochondrial ATP synthase catalyzes ATP synthesis in oxidative phosphorylation. This complex consists of two parts: the F_0 part which is embedded in the membrane and the F_1 part which is able to bind nucleotides and hydrolyze ATP. The β -subunit of the F_1 part contains the catalytic sites although it has been established that both the α and β subunits are essential for the enzyme activity [31]. In the present proteomic study, while the expression of the β -subunit was increased, the expression of the α -subunit was reduced in the left ventricle of SHR. These findings may be in accordance with the reduced ATP levels that have been reported in the hypertrophied hearts [32].

Creatine kinase

Creatine kinase M chain was found upregulated in the proteomic map of the left ventricle of SHR. This observation may be important since under conditions when ATP demand exceeds ATP supply, as it may occur in the hypertrophied heart, creatine kinase reaction is one of the main mechanisms that the heart uses to maintain ATP levels. However, although we have detected an increased creatine kinase expression in the left hypertrophied ventricle of SHR, previous studies have reported that the total creatine pool is as much as 60% lower in left ventricular hypertrophy in different animal models [33]. Moreover, a recent study of Smith et al has reported that the net ATP flux through creatine kinase is reduced in human left ventricular hypertrophy [34]. Then, we should be cautious with this finding, since the increase in creatine kinase may be not enough to compensate ATP synthesis due to the reduction of creatine in the hypertrophied heart.

Study limitations

As study limitation, here we determine only changes in the protein expression. Therefore, we should be careful with the results interpretation since changes in the level of protein expression are not the only fact to assess different protein activity. In this regard, the ultimate impact of the changes observed in the expression of proteins can be affected by several other factors including availability of substrate, protein functional integrity and disposition of their products among other conditions/factors.

It should be emphasized that alterations in energy metabolism have emerged as important factors for the development of poor contractile function in hypertrophied hearts, and the subsequent heart failure. Thus, the use of new technologies, such as proteomics, allowed us to analyze more in depth, and in an integrated form, the metabolic alterations associated with hypertensive cardiac hypertrophy. In fact, under the present experimental conditions, several proteins involved in the main energy metabolic pathways (fatty acid β -oxidation, glycolysis, tricarboxylic acid cycle and mitochondrial oxidative phosphorylation) were up and downexpressed, further supporting that left ventricle hypertrophy associated with mild diastolic dysfunction, is a transitory situation towards more complete energy metabolic deficiencies present in heart failure and probably neither fatty acid β -oxidation nor glycolysis are the only sources for energy in the hypertrophied left ventricle.

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