Identification of transthyretin and β4-thymosin as potential biomarkers in acute coronary syndrome by two independent methods, 2-DE/DIGE and SELDI-TOF

*Ciordia, S.[a], Moral, V.[b], De los Ríos, V.[a] , Barderas M. G.[b,c], de la Cuesta F. [b], Tarín N[d], Tuñon J [e], Jimenez-Nacher J. J.[f], Lopez-Bescos L[f], Egido J[g], *Vivanco, F.[b] Albar, J. P.[a]*

[a]Unidad de Proteómica, Centro Nacional de Biotecnología/CSIC, Campus de Cantoblanco, E-28049 Madrid. Spain, [b]Servicio de Inmunología. Fundación Jiménez Díaz. Madrid, [c]Departamento Fisiopatologia Vascular, Hospital Nacional de Paraplejicos, SESCAM, Toledo. Spain, [d]Departamento Cardiología, Hospital de Fuenlabrada, Madrid, Spain, [e]Departamento Cardiología, Fundacion Jimnez Diaz, Madrid, Spain, [f]Departamento Cardiología, Fundacion Hospital de Alcorcon, Madrid, Spain, [g]Departamento de Patología Renal, Fundacion Jiménez Diaz, Madrid, Spain

Acute myocardial infarction (AMI) is one of the leading causes of death in the world and remains a complex pathophysiologic process involving inflammatory, hemostatic and vascular processes. We employed two independent and complementary approaches, SELDI-TOF, and 2-DE/ DIGE in a first phase exploratory biomarker study to analyze modifications in the serum protein map during an acute coronary syndrome (ACS); It disclosed that the levels of two proteins, transthyretin (TTR; 14000 m/z) and acetylated-ß4-thymosin (4970 m/z) were significantly altered in acute coronary syndrome patients in comparison with healthy subjects. TTR was identified by 2-DE/DIGE and SELDI-TOF and confirmed by Western blotting whereas ß4- thymosin was detected only by SELDI-TOF owing to its low molecular mass and confirmed by Western blotting. Whereas TTR is involved in the transport of various biologically active compounds ß4- thymosin is essential for cardiomyocyte survival, cardioprotection and repair in the adult heart. Identification of both proteins could help in the understanding of the basis for allowing the diagnosis to be made at an earlier stage of the disease when the treatment is possible.

Weight loss and protein expression profiles in the *Gastrocnemius* muscle of two rabbit breeds

Almeida AM[1,2], van Harten S[1], Campos A[2], Varela A[2,3], Alfaro L.[1]

[1]Instituto de Investigação Científica Tropical, Lisboa, Portugal. aalmeida@fmv.utl.pt; [2]Instituto de Tecnologia Química e Biológica, Oeiras, Portugal; [3]Universidade de Évora, Évora, Portugal

Introduction

Seasonal Weight Loss (SWL) poses a serious limitation to animal production in Tropical and Mediterranean climates, strongly conditioning agriculture in these areas (Almeida et al., 2006). The study of the physiological and molecular mechanisms by which domestic animal breeds respond to SWL is of capital interest with important implications in animal selection schemes. Recently, the use of proteomic has allowed a much greater insight on the molecular mechanisms at the protein level in a vast array of physiological systems (Jia et al., 2007. The goal of this study is to determine differential protein expression in the muscle of two rabbit breeds that show different tolerance to SWL.

Material and methods

Six Wild (WR) and eight New Zealand White (NZ) rabbits (*Oryctolagus cuniculus*), were used in this trial. Animals of each breed were divided into two weight-matched experimental groups: C (Control – fed *ad libitum*) and R (Restricted fed to 30% *ad libitum*). Animals were fed on commercial pellets (Biona 701) and experiment lasted 30 days. After euthanasia, the *gastrocnemius* muscle was excised. European Union Regulations on animal experimentation were followed. Total protein was extracted (Jia et al., 2007) and their concentrations determined using PlusOne 2D Quantification Kit (GE Healthcare). A total of 20 µg of protein were separated by standard 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Gels were stained using Colloidal Coomasie Blue (Neuhoff et al., 1988). After digitalization, the relative intensity of each individual band of each lane was calculated using LabImage 1D 2006 software (Kapelan technologies, Germany - www.labimage.net). Band intensities with the same molecular weight, were statistically compared using single-factor analysis of variance. Differentially expressed proteins were identified using PMF (Peptide Mass Fingerprinting). Proteins were in-gel digested (Pandey et al., 2000). Peptide mixture was purified and concentrated by R2 pore microcolumns (Gobom et al., 1999) and eluted directly to the MALDI plate with 0.5 µl of matrix α-cyano-4-hydroxycinnamic acid (CHCA) (5mg/ml) prepared in 70% (v/v) acetonitrile with 0.1% (v/v) TFA. The *m/z* spectra were acquired in a Voyager-DE STR MALDI-TOF mass spectrometer. Protein identification was achieved in MASCOT search engine (www.matrixscience.com), in MSDB databases.

Results

Animals in the restricted groups lost 20% of the initial body weight, whereas control animals maintained the initial weight levels. SDS-PAGE results of the total protein extracts are presented in Figure 1.

Proteins bands that showed to have different expression in the four experimental groups are indicated (P37, P39, P311, P312, P314 and P35) as well as other identified proteins that showed to have the same expression in the four experimental groups (P34, P38 and P38b). Relative intensities of each of the above mentioned proteins are presented in table 1. Table 2 shows the identification details of each of the proteins relevant to this study.

Table 1. Protein expression level comparison

Protein Reference	NZR	NZC	WRR	WRC
P35	2.53^{a} (0.12)	4.80^{b} (0.55)	4.21^{b} (0.74)	3.80^{b} (0.13)
P37	4.96^{a} (0.55)	4.59^{a} (3.4)	Absence	3.88^{a} (2.7)
P39	4.81^{a} (0.40)	6.06^{a} (1.90)	3.73^{a} (0.03)	Absence
P311	Absence	1.99^{a} (0.20)	Absence	1.98^{a} (0.6)
P312	Absence	2.47^{a} (0.48)	Absence	3.76^{a} (0.03)
P314	Absence	1.48^{a} (0.08)	Absence	2.54^{a} (0.26)
P34	7.81^{a} (0.96)	7.71^{a} (1.03)	6.53^{a} (4.02)	7.39^{a} (1.40)
P38	22.66^{a} (0.15)	20.06^{a} (5.38)	19.54^{a} (4.50)	19.17^{a} (0.68)
P38b	5.88^{a} (0.67)	4.05^{a} (1.19)	5.82^{a} (0.34)	4.77^{a} (0.46)

Results are shown in abstract values - Relative Intensities; Variances are shown between parenthesis; ^{a,b} – Rows with different superscripts indicate statistical significance ($p<0.05$); WRR (Wild Rabbits Restricted); WRC (Wild Rabbit Control); NZR (New Zealand White Restricted) and NZC (New Zealand White Control)

Table 2. Protein Identification

Protein Reference	Peptide search/ match	Theoretical Molecular Weight (Kda)/pI	Coverage (%)	Score (significance level)	Protein	Species
P35	33/13	57/7.70	29	105(68)	Pyruvate kinase chain A	*Oryctolagus cuniculus*
P37	33/8	47.2/7.75	29	72(68)	Phosphopyruvate hydratase β	*Oryctolagus cuniculus*
P39	23/8	33.8/8.76	37	86(68)	AAD32624	*Rattus sp.*
P311	40/8	20/6.76	36	86(68)	α-crystalin chain B	*Oryctolagus cuniculus*
P312	18/9	17/4.56	38	94/(78)	Myosin light chain slow skeletal muscle	*Oryctolagus cuniculus*
P314	26/8	16.5/4.62	41	72/(68)	Myosin light chain 3, skeletal muscle isoform	*Oryctolagus cuniculus*
P34	37/18	96/6.57	25	144(68)	2GPB glycogen phosphorylase	*Oryctolagus cuniculus*
P38	21/10	41/5.16	37	121(68)	1ALMV actin, chain V	*Oryctolagus cuniculus*
P38b	32/9	35.9/6.90	24	72/(68)	Glycerladehyde-3-phosphate dehydrogenase	*Sus scrofa*

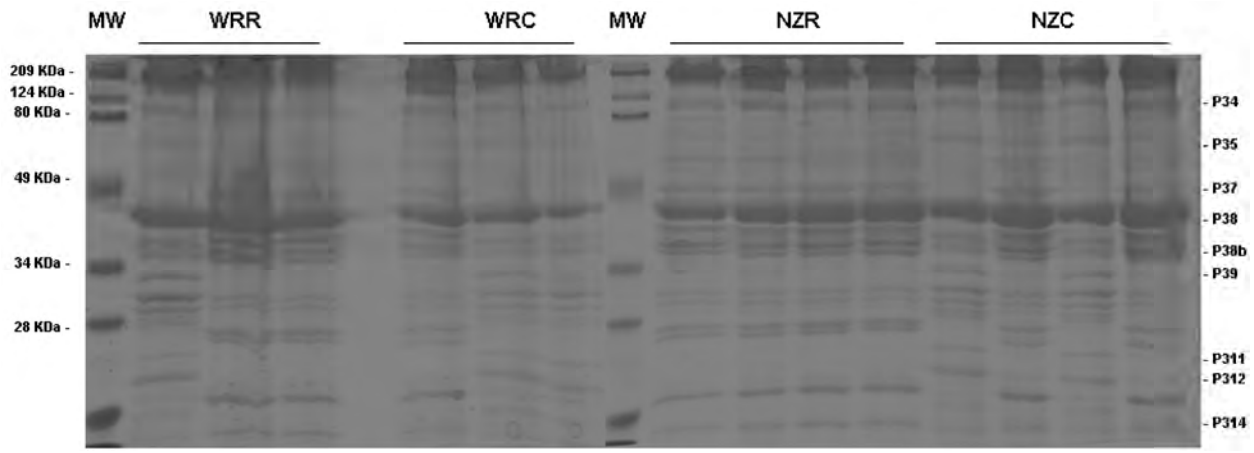

***Figure 1** – Electrophoresis of total protein extracts from the gastrocnemius muscle of the experimental groups: WRR (Wild Rabbits Restricted); WRC (Wild Rabbit Control); NZR (New Zealand White Restricted) and NZC (New Zealand White Control). Molecular Weight Markers are also presented (MW), as well as proteins differentially expressed (P37, P39, P311, P312, P314 and P35) and four identified proteins with the same level of expression (P34, P38 and P38b). Staining performed with colloidal blue.*

Conclusions

From this the following major conclusions can be ascertained:

1) Proteins P311 (α-crystalin chain B), P312 (Myosin light chain slow skeletal muscle) and P314 (Myosin light chain 3, skeletal muscle isoform) can be considered as markers of weight loss as they were not found in detectable levels in restricted-fed groups. Proteins P34 (glycogen phosphorylase), P38 (Actin chain V) and P38b (Glycerladehyde-3-phosphate) were statistically similar for all experimental groups indicating lack of use as markers of weight loss;

2) Pyruvate kinase (P35 - PK) is a marker of sarcopenia (loss of muscle mass due to old age, Doran, 2007). WRR group showed similar PK levels to those of control groups indicating that there are muscle energy resources being used in the NZR and not in the WRR (less muscle mass) thus representing a NZ tolerance to energy restricted diets.

3) Myosin Light chains family (P312, P314) seems to be severely affected by undernutrition in both rabbit breeds an indication that weight loss affects muscle structure at the levels where those proteins are located;

4) This preliminary study indicates that several muscle proteins can be used as valid markers for weight loss and undernutrition, hence an important role as tool in studying breed adaptation to SWL. However, one-dimensional gel electrophoresis has a strong limitation regarding protein overlapping within the same band and low amounts of protein are loaded per lane strongly limiting the number of proteins that can be identified as markers of SWL. It is therefore desirable to conduct a similar experiment using two-dimensional gel electrophoresis and peptide mass fingerprinting.

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