ORIGINAL

Proteomic analysis of the heart in normal aging mice

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Abstract : Aging induces pathological cardiovascular changes such as cardiac dysfunction and arteriosclerosis. With aging, heart cells, especially, become more susceptible to lethal damage. In this report, we tried to understand the precise mechanism of myocardial change resulting from aging by examining the heart proteome in aging mice using two-dimensional gel electrophoresis (2DE). The proteins were stained with fluorescence dyes (SYPRO Ruby and Pro-Q Diamond) and identified by subsequent MALDI-TOF-MS/MS. As a result, markedly altered levels of 14 proteins and 7 phosphoproteins were detected in the hearts of 3-, 7-, 11-, and 20-month-old mice. The functions of these identified proteins and phosphoproteins were energy metabolism, muscle contraction, glycolysis, and cytoskeletal support. Additionally, the results of Western blotting confirmed changes in the expression of FTH, CPNE5, and SUCLA2. These findings showed that aging modified the expression of proteins and phosphoproteins in the heart. We suggest that changes in the expression of these proteins are critical to the development of cardiac dysfunction resulting from aging. J. Med. Invest. 69 : 217-223, August, 2022

Keywords **:** *Proteome, phosphoprotein, heart, aging, mitochondrial protein*

INTRODUCTION

Aging, a prominent factor influencing the development of physiological and metabolic dysfunction, leads to structural and functional change in all organisms and multiple organs. In particular, aging-dependent dysfunction of the circulatory system including heart and vessels is directly linked to death.

Aging-induced deterioration of cardiac functions, such as diminished receptor reactivity of the heart (1), reduction of peak filling rate (2), cardiac hypertrophy (3), and endothelial dysfunction (4) lead to heart failure and cardiogenic shock ending in death at age 60–80 years (5). For prevention of heart failure, it is indispensable to understand the molecular mechanism of aging-related myocardial dysfunction.

It has been thought that mitochondrial damage is key to the aging process, especially in the myocardium which has high oxidative phosphorylation activity. Aging-related decline in cardiac mitochondrial function and ATP production (6, 7) has been shown to underlie the development of ischemic heart disease and heart failure (8). Mitochondria have important roles in maintaining energy metabolism in cardiac cells. Furthermore these processes are regulated by protein phosphorylation by kinase/phosphatase (9).

Our goal was to understand the systems underlying cardiac aging. To achieve that goal, we examined changes in heart protein and heart phosphoprotein expression in 3-, 7-, 11-, and 20-month-old C57BL/6 male mice using protein separation by two-dimensional electrophoresis (2DE), visualization by SYPRO Ruby and Pro-Q Diamond fluorescence dye staining, respectively, and identification by MALDI-TOF-MS/MS.

Received for publication February 17, 2022 ; accepted April 10, 2022.

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MATERIALS AND METHODS

Materials

The reagents and materials of 2DE and Western blotting were described previously (10). The following antibodies were purchased from the indicated companies, rabbit anti-FTH (Cell Signaling Technology Inc., Danvers, MA, USA), rabbit anti-CPNE5, rabbit anti-SUCLA2 (Abcam, Cambridge, UK), and goat anti-rabbit IgG (Fc)–horseradish peroxidase conjugate (Promega Corporation, Madison, WI, USA).

Animal subjects

Animal breeding and handling were described previously (10). Animal breeding and handling were done strictly according to the Guidelines for Animal Experimentation at Kobe Gakuin University and Himeji Dokkyo University.

Preparation of heart tissue samples

C57BL/6 mice were sacrificed at 3, 7, 11 and 20 months, and their hearts were isolated and washed three times in cold PBS. The preparation of the mouse tissue was performed as described previously (11) with minor modifications. The heart tissue was immersed in 800 µl of lysis buffer in a 2-ml tube with a 5-mm metal bead and disrupted four times with a beads-crusher type homogenizer µT-12 (TAITEC CORPORATION, Kyoto, Japan) and repeated shaking (3200 r/min, 30 s). After centrifugation at 15,000 × g for 30 min at RT, the supernatant was subjected to 2DE.

Two-dimensional electrophoresis

The 2DE was performed as described previously (11). In brief, 500 µg of protein was loaded on to Immobiline DryStrip gels (IPG strips, GE Healthcare Life Sciences, MA, USA). After they were reduced and alkylated, the IPG strips were subjected to SDS–PAGE with escalating voltage in three steps (50 V for 6 h, 100 V for 6 h and 2000 V for 6 h).

SYPRO Ruby or Pro-Q Diamond staining

The SYPRO Ruby protein or Pro-Q Diamond phosphoprotein staining was carried out as described previously (10). In brief, after 2DE, the gels were fixed, washed, and then stained with SYPRO Ruby or Pro-Q Diamond. Images of the stained gels were captured using a FluoroPhorestar 3000 digital image capture system (Anatech, Tokyo, Japan).

Image analysis

Following image acquisition, differences in heart protein composition among the 3-, 7-, 11-, and 20-month-old mice were detected using Prodigy SameSpots software (TOTALLAB, Newcastle, UK) to identify differences in fluorescence signals among the gels. Heart protein composition changed over time, and the differences in composition between timepoints were statistically analyzed by ANOVA with a significance level of 95%.

MALDI-TOF-MS/MS and protein identification

MALDI-TOF-MS/MS analysis were carried out as described previously(10). In brief, aliquots (1.0 μl) of digested material and matrix were spotted onto a plate for mass spectra analysis in a MALDI-TOF-MS/MS analyzer. All of the MS measurement conditions were previously described (10).

Western blotting

Western blotting was performed as previously described(10). Briefly, the denatured mouse heart proteins (10 μg) were applied to gels. After PAGE, the separated proteins were blotted on polyvinylidene fluoride (PVDF) membranes using a transblotter. The membranes were blocked with non-fat dried milk (5% in TBS) and incubated first with following antibodies : CPNE5 (diluted 1 : 1000), FTH (diluted 1 : 1000), SUCLA2 (diluted 1 : 250), GAPDH (diluted 1 : 1000), and then with peroxidase-conjugated goat anti-rabbit or anti-mouse immunoglobulin (diluted 1 : 10,000). The signals of chemiluminescence and images were captured using a VersaDoc MP 5000 system (Bio-Rad Laboratories, Inc., Hercules, CA) for chemiluminescence detection.

RESULTS

Changes in murine cardiac protein and phosphoprotein expression were assessed at 3, 7, 11, and 20 months by Prodigy SameSpots software and identified by MALDI-TOF-MS/MS. Image analysis detected approximately 330 protein spots (SYPRO Ruby-stained gels, Fig. 1 : A-D) and approximately 250 phosphoprotein spots (Pro-Q Diamond-stained gels, Fig. 2 : A-D). The spots on 2DE gels, which represented the proteins (Fig. 3A) and phosphoproteins (Fig. 3B) with changed levels, were

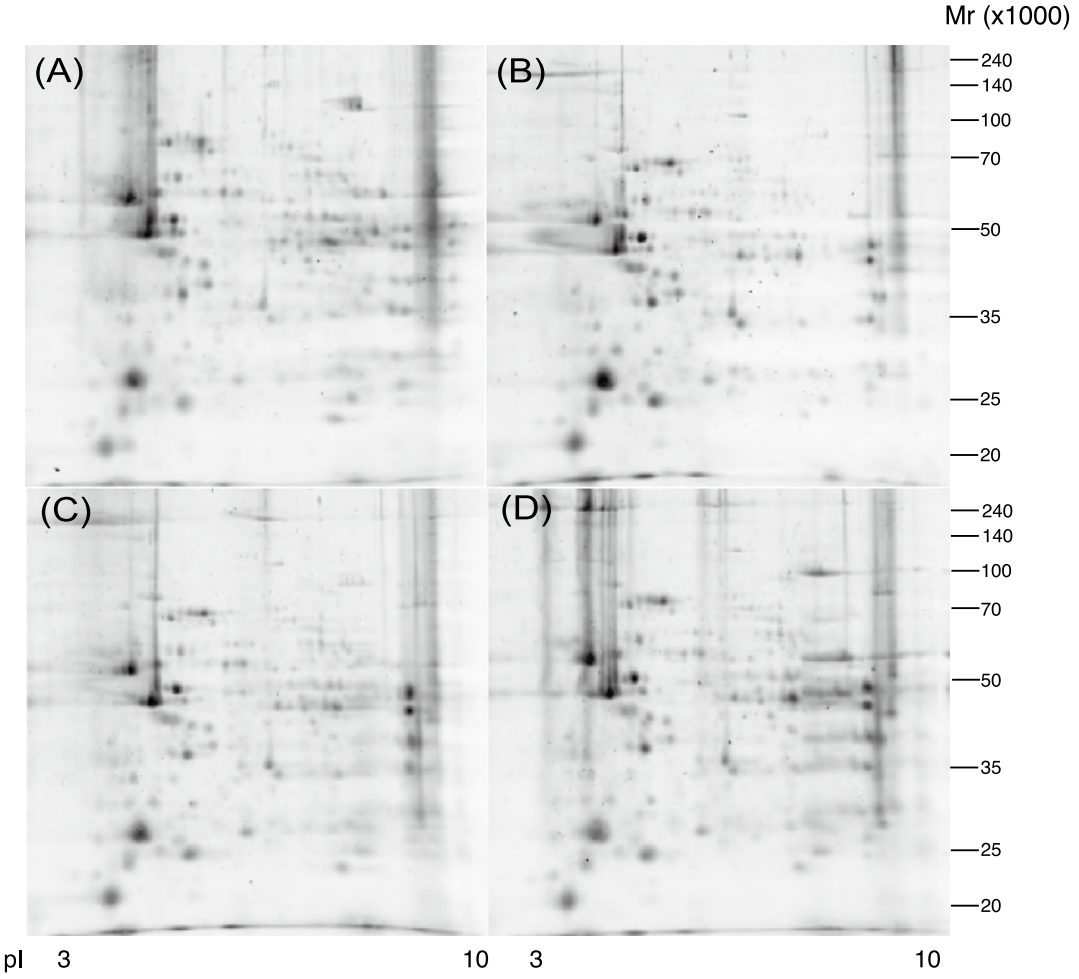

Fig 1. Detection of protein expression in representative SYPRO Ruby-stained 2DE gels of heart proteins from (A) 3-, (B) 7-, (C) 11-, and (D) 20-month-old mice. Each gel was loaded with 500 μg of protein.

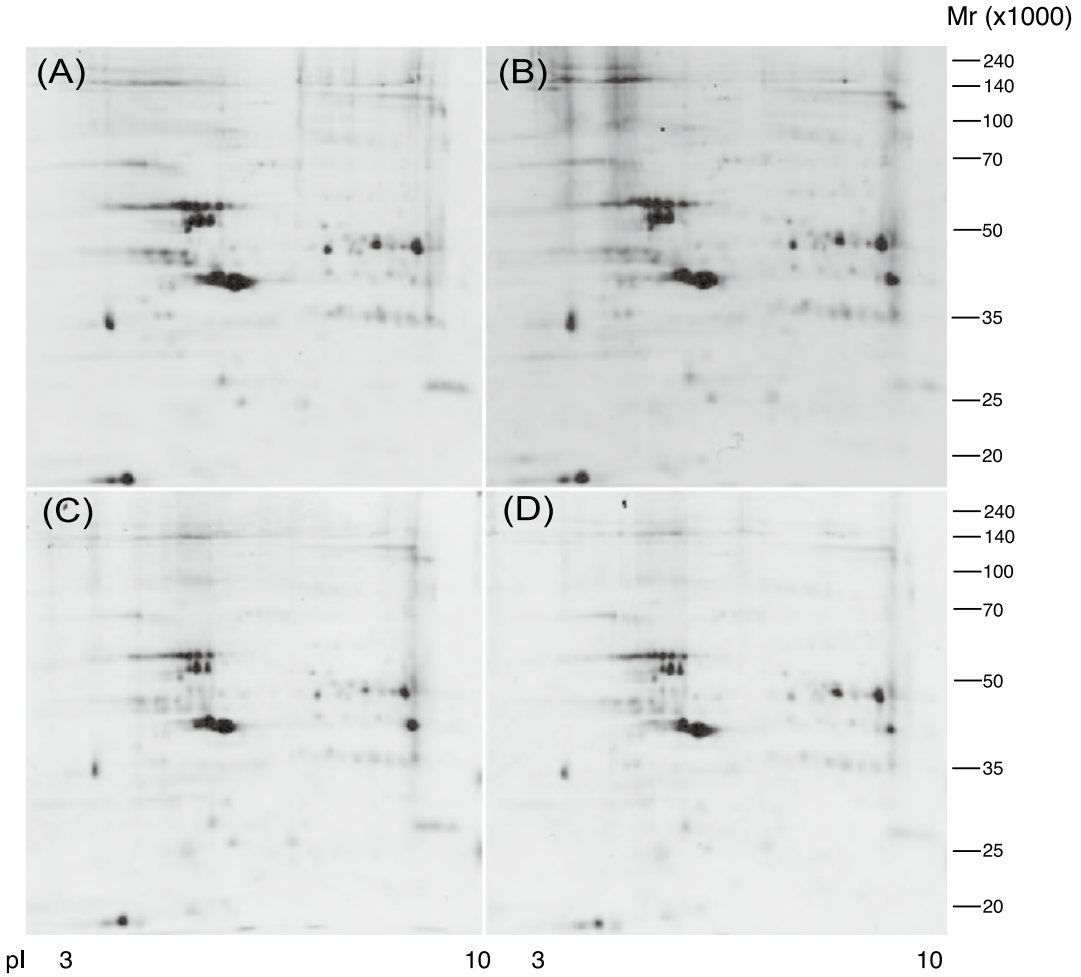

Fig 2. Detection of protein expression in representative Pro-Q Diamond-stained 2DE gels of heart proteins from (A) 3-, (B) 7-, (C) 11-, and (D) 20-month-old mice. Each gel was loaded with 500 μg of protein.

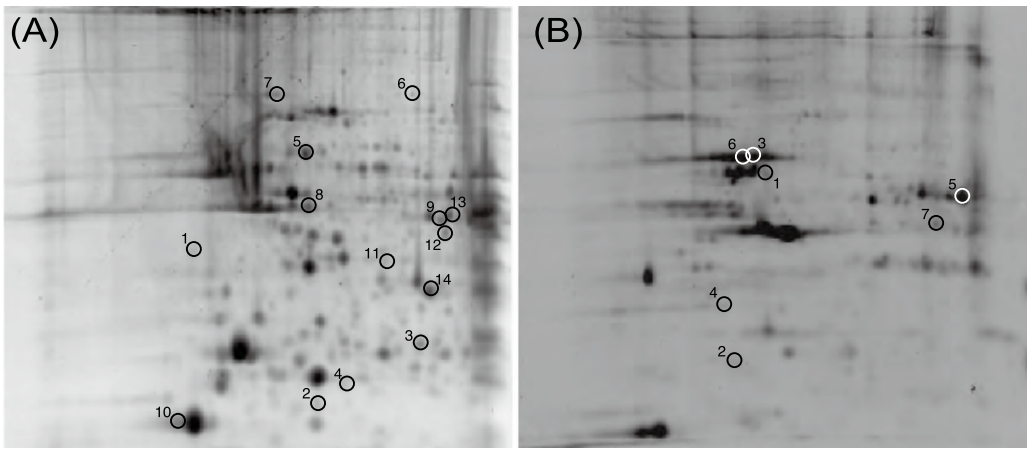

Fig 3. A matched group of heart proteins that differentially changed in abundance with aging in mice. The proteins in the gel were stained by (A) SYPRO Ruby and (B) Pro-Q Diamond. The numbers are same as in Tables 1 and 2.

analyzed and quantified using the Prodigy SameSpots program. The brightness of signals denoting different cardiac proteins from naturally aging mice indicated alterations in the levels of 47 proteins (ANOVA<0.05, fold >1.1 or <0.9) and 12 phosphoproteins (ANOVA<0.05, fold >1.1 or <0.9). Finally, 14 polypeptides were identified as proteins (Table 1) and 7 polypeptides were identified as phosphoproteins (Table 2).

Identification of proteins with changed expression in the hearts of normal aging mice

The levels of myosin light chain (MLC), isocitrate dehydrogenase subunit alpha (IDH3A), glial fibrillary acidic protein (GFAP), ATP-specific succinyl-CoA synthetase beta subunit (SUCLA2), and Acyl-CoA thioesterase 2 (ACOT2) were significantly decreased over the 3–20 month period of observation, while levels of ferritin heavy chain (FTH), copine-5, and myosin light chain 2 (MLC-2) were increased. Those with fluctuating levels over the same period included heat shock protein 27 (HSP27), NADH dehydrogenase (ubiquinone) Fe-S protein 1, isovaleryl-CoA dehydrogenase (Ivd), pyruvate dehydrogenase E1 component subunit alpha (Pdha1), short chain acyl-CoA dehydrogenase, and delta (3,5)-Delta (2,4)-dienoyl-CoA isomerase (Ech1) (Table 1).

Identification of cardiac phosphoproteins that changed expression during normal aging

Expression was significantly increased for desmin, NADH dehydrogenase iron-sulfur protein 8, alpha-actin, ubiquinone biosynthesis protein COQ9, pyruvate dehydrogenase E1 component subunit alpha, and alpha-cardiac actin, and significantly decreased for NADH dehydrogenase1 alpha subcomplex subunit 10 (Table 2).

Validation of FTH, CPNE5, and SUCLA2 expression by Western blotting

We performed Western blotting to verify the level of expression of FTH, CPNE5, and SUCLA2 and used chemiluminescence intensity to measure the relative amounts of each protein by ImageJ software analysis. The expressions of FTH and CPNE5 in the hearts of naturally aging mice increased from their levels at 3 months to their levels at 7, 11, and 20 months (Fig. 4-A, B). The expression of FTH was increased 3.0 fold at 7 months and 2.6 fold at 11 months (Fig. 4-A) and that of CPNE5 was increased 2.3 fold at 11 months (Fig. 4-B). The expression of SUCLA2 was decreased at 20 months compared to its expression at 7 and 11 months (Fig. 4-C). Western blotting validated the changes in FTH, CPNE5, and SUCLA2 levels, including the tendency of

Table 1A. Differentially expressed heart proteins in normal aging mice divided into 3-, 7-, 11-, and 20-month-old groups (n = 3).

Spot No.	Description	Gene ID[a]	Score[b]	SC[c](%)	Theoretical[b] MW	pI	Fold 7M	11M	20M
1	Myosin light chain 1/3	29789016	50	16	20695	4.98	0.57	0.68	0.70
2	Ferritin heavy chain	6753912	51	10	21224	5.53	1.31	1.19	1.51
3	Heat shock protein HSP27	424143	44	12	22944	6.12	1.48	0.61	1.15
4	Copine-5	23346611	69	48	65592	5.49	1.24	1.08	1.16
5	Isocitrate dehydrogenase [NAD] subunit alpha	18250284	234	16	39638.7	6.27	0.90	0.87	0.88
6	Glial fibrillary acidic protein	51066	41	12	48494	5.29	0.59	0.98	0.80
7	NADH dehydrogenase (ubiquinone) Fe-S protein 1	13879366	48	3	79748.7	5.51	0.64	1.44	0.99
8	ATP-specific succinyl-CoA synthetase beta subunit	3766201	121	7	46244.5	5.65	0.89	0.88	0.82
9	Isovaleryl-CoA dehydrogenase	9789985	56	8	46325.4	8.53	1.07	0.92	0.77
10	Myosin light chain 2	199985	92	18	18767.3	4.75	1.09	1.07	1.14
11	Pyruvate dehydrogenase E1 component subunit alpha	6679261	81	10	43231.6	8.49	1.11	1.21	0.82
12	Short chain acyl-CoA dehydrogenase	192659	63	8	44946.7	8.96	0.92	1.05	0.80
13	Acyl-CoA thioesterase 2	39992610	62	6	49641	7.21	0.96	0.90	0.94
14	Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase	7949037	158	16	36118.5	7.6	1.12	0.97	0.88

The proteins of mouse heart were analyzed by 2DE and MALDI-TOF MS/MS.
[a]The gene ID accession numbers (GI acc. no.) in the Swiss Prot database of proteins identified as spots on 2-DE gels (see Figure 1).
[b]Score relates to the probability assignment ; molecular weight and pI are theoretical.
[c]Score and sequence coverage (SC) were calculated by the MASCOT search engine (http://www.matrixscience.com).
"Fold" was calculated relative to the protein's expression level at 3 months.
Data were analyzed by ANOVA.

Table 1B. Cellular/subcellular localization and function of proteins differentially expressed due to aging.

Spot No.	Protein	Cellular/subcellular location	Function
1	Myosin light chain	Cytoplasm, myofibrils	Muscle contraction
2	Ferritin heavy chain	Secreted protein	Bind and transport iron
3	Heat shock protein HSP27	Cytoplasm, cytoskeleton, nucleus	Chaperone
4	Copine-5	Extracellular	Cellular response to calcium ion
5	Isocitrate dehydrogenase [NAD] subunit alpha	Mitochondria	Tricarboxylic acid cycle
6	Glial fibrillary acidic protein	Cytoplasm	Intermediate filament
7	NADH dehydrogenase (ubiquinone) Fe-S protein 1	Mitochondria	Electron transport
8	ATP-specific succinyl-CoA synthetase beta subunit	Mitochondria	Tricarboxylic acid cycle
9	Isovaleryl-CoA dehydrogenase	Mitochondria	Fatty acid beta-oxidation using acyl-CoA dehydrogenase
10	Myosin light chain 2	Cytoplasm, myofibrils	Muscle contraction
11	Pyruvate dehydrogenase E1 component subunit alpha	Mitochondria	Glycolysis
12	Short chain acyl-CoA dehydrogenase	Mitochondria	Fatty acid metabolic process
13	Acyl-CoA thioesterase 2	Peroxisome	Acyl-CoA metabolic process
14	Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase	Mitochondria	Fatty acid beta-oxidation

Table 2A. Differentially expressed heart phosphoproteins in naturally aging mice divided into 3-, 7-, 11-, and 20-month-old groups (n = 3).

Spot No.	Description	Gene ID[a]	Score[b]	SC[c](%)	Theoretical[b] MW	pI	Fold 7M	11M	20M
1	Desmin	13346	379	20	53522	5.21	1.02	1.76	1.35
2	NADH dehydrogenase iron-sulfur protein 8	225887	300	21	24479	5.89	1.18	1.18	1.13
3	Alpha-actin	11475	134	11	38016	5.45	1.11	1.24	1.09
4	Ubiquinone biosynthesis protein COQ9	67914	194	14	35232	5.6	0.93	1.40	1.06
5	Pyruvate dehydrogenase E1 component subunit alpha	18598	103	12	43888	8.5	1.07	1.16	1.19
6	Alpha-cardiac actin, partial	11464	265	23	42043	5.23	1.08	1.17	1.14
7	NADH dehydrogenase1 alpha subcomplex subunit 10	67273	175	12	40863	7.63	0.99	0.79	1.01

The proteins of mouse heart were analyzed by 2DE and MALDI-TOF MS/MS.
[a]The gene ID accession numbers (GI acc. no.) in the Swiss Prot database of proteins identified as spots on 2-DE gels (see Figure 2).
[b]Score relates to the probability assignment ; molecular weight and pI are theoretical.
[c]Score and sequence coverage (SC) were calculated by the MASCOT search engine (http://www.matrixscience.com).
"Fold" was calculated relative to the protein's expression level at 3 months.
Data were analyzed by ANOVA.

Table 2B. Cellular/subcellular localization and function of phosphoproteins differentially expressed due to normal aging.

Spot No.	Protein	Cellular/subcellular location	Function
1	Desmin	Nucleus	Regulation of heart contraction
2	NADH dehydrogenase iron-sulfur protein 8	Mitochondria	Mitochondrial respiratory chain complex I assembly
3	Alpha-actin	Cytoskeleton	Muscle contraction
4	Ubiquinone biosynthesis protein COQ9	Mitochondria	Mitochondrial electron transport
5	Pyruvate dehydrogenase E1 component subunit alpha	Mitochondria	Acetyl-CoA biosynthetic process from pyruvate
6	Alpha-cardiac actin, partial	Cytoskeleton	Heart contraction
7	NADH dehydrogenase1 alpha subcomplex subunit 10	Mitochondria	Mitochondrial electron transport

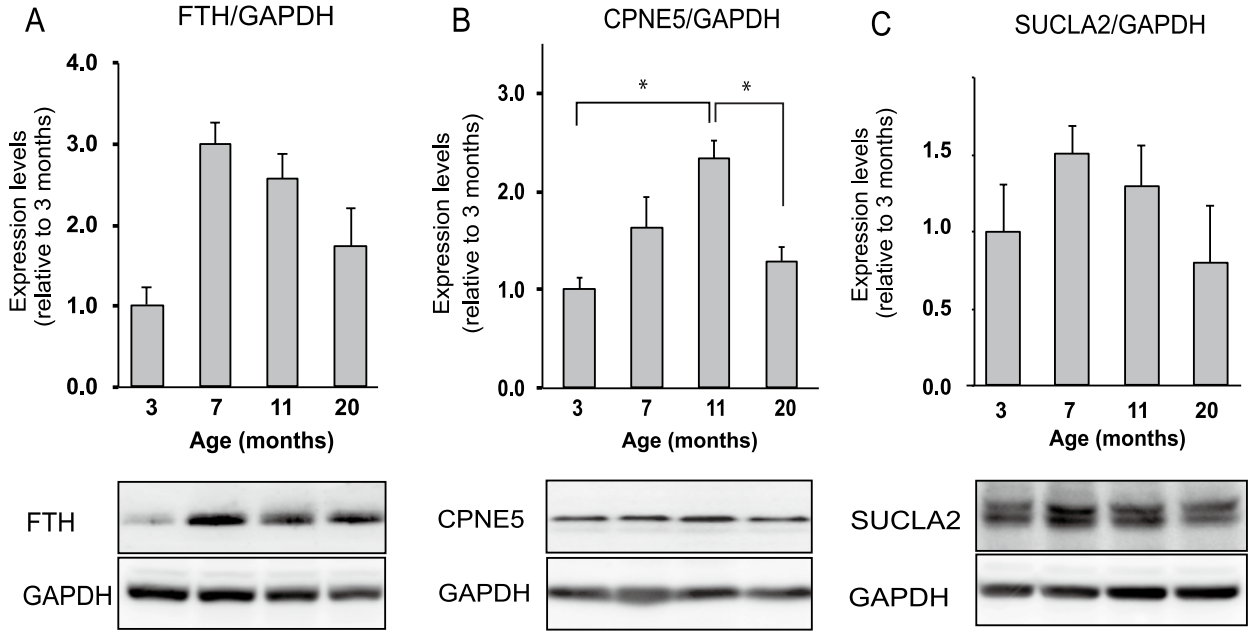

Fig 4. Western blotting of (A) FTH, (B) CPNE5, and (C) SUCLA2 from the hearts of 3-, 7-, 11-, and 20-month-old mice. FTH : ferritin heavy chain, CPNE5 : copine-5, SUCLA2 : ATP-specific succinyl-CoA synthetase beta subunit. GAPDH was used as the internal control protein. Data are presented as mean ± S.E.M. Data were analyzed by ANOVA with Tukey's post-hoc test and multiple comparisons. ; * $P < 0.05$ among 3-, 7-, 11-, and 20-month-old mice.

FTH and CPNE5 to increase with normal aging (Fig. 4-A, B). Proteomic analysis showed that SUCLA2 expression tended to decrease. However, Western blotting showed that SUCLA expression reached a peak at 7 months, when it was 2.2 times higher than at 3 months, and then gradually decreased during the subsequent 13-month period (Fig. 4-C).

DISCUSSION

In this report, 14 heart proteins and 7 heart phosphoproteins whose expression changed with normal aging in mice were identified by 2DE and MS/MS. Furthermore, changes in the levels of some of the cardiac proteins were verified by Western blotting (Fig. 4). These proteins were categorized functionally using the UniProtKB (http://www.uniprot.org/uniprot/) database (Table 1B, 2B). Our results showed that expression of proteins in the MLC family was changed in aging mice. Especially, MLC1/3 was drastically decreased, but MLC-2 was slightly increased from age 7 months to age 20 months (Table 1A). The MLC proteins are classified into two groups, phosphorylatable MLCs (MLC-2-type, or regulatory light chains) and alkali MLCs (MLC-1-type or essential light chains) (12). MLCs play a potential role in heart development. The mutations of the human myosin regulatory light chain 2 (*MYL2*) gene were linked to hypertrophic cardiomyopathy (13, 14). The roles and mechanisms of MLCs expression in aging heart are still unclear ; however, the downregulation of MLC1/3 expression is closely related to the dysfunction of the aging heart.

Aging is closely linked to energy metabolism pathways (such as glycolysis, TCA cycle, and the electron transport chain) (15). As is evident from Table 1B and 2B, we identified ten protein spots that corresponded to mitochondrial proteins. Mitochondria supply high energy phosphates needed for heart contraction and relaxation. Mitochondria are dysfunctional in many heart diseases(16), and our results also suggested that aging affects the expression of mitochondrial proteins in the heart. In our study, levels of IDH, SUCLA2, ACADS, and ACOT2 were reduced in the hearts of aging mice. In addition, isovaleryl-CoA dehydrogenase (Ivd), pyruvate dehydrogenase E1 (Pdha1), and delta (3,5)-Delta(2,4)-dienoyl-CoA isomerase (Ech1) were decreased in mice aging from 3 months to 20 months. Among these mitochondrial proteins, some were involved in glycolysis (such as Pdha1), the TCA cycle (such as IDH, SUCLA2), and the electron transport chain (such as SUCLA2). We have studied the expressions of four enzymes in the fatty-acid β-oxidation pathway (Ivd, ACADS, ACOT2, and Ech1), all of which decreased as the heart aged.

Ferritin acts as a regulator of intracellular iron metabolism and supplies iron to mitochondria (17, 18). We observed an increase in FTH expression at age 20 months from its level at age 3 months (Table 1A, Fig. 4). An increase in the amount of ferritin with aging causes accumulation of excess iron in mitochondria, and it is possible that the reactive oxygen species (ROS) generated as a result may cause mitochondrial dysfunction.

Furthermore, FTH, CPNE5, and SUCLA2 showed an increasing trend in the 7- and 11-month-old hearts, however decreased to the same level at 20 months of age as at 3 months of age (Fig. 4). Although these proteins may be associated with the aging process in the heart, they do not show sustained increases or decreases with aging and are not suitable as markers or indicators of cardiac aging.

In addition, protein phosphorylation is a major post-transcriptional modification affecting many intracellular functions (19). In the present study, Pro-Q Diamond staining in 2D-gel electrophoresis showed a change in the levels of seven heart phosphoproteins with aging from 3 months to 20 months in mice. Phosphorylated desmin, especially, was drastically increased in expression during aging. Desmin is the main intermediate filament protein of smooth, skeletal, and cardiac muscle (20). Several lines of evidence closely link increased expression of phosphorylated desmin to heart failure. Notably, Pinet *et al*. showed that levels of phosphorylated and cleaved desmin were increased in a heart failure animal model and in humans with heart failure (21). Our result and the above evidences prove that desmin phosphorylation is an important aging-related factor in heart failure.

In our study, the expression of phospho-alpha-cardiac actin tended to increase (Table 2A), although actin binding of cardiac myosin binding protein is reduced by phosphorylation (22). The effect of direct phosphorylation of actin remains unclear.

Because changes in the proteome and phospho-proteome of the aging mouse heart could be attributable to aging and pathophysiological processes linked to heart diseases, there is the possibility that using the technology of proteomics to investigate changes in the expression of heart proteins and heart phosphorylated proteins with aging will reveal some of the mechanisms of aging and cardiac dysfunction in the future.

ACKNOWLEDGEMENTS

This work was supported by a grant from Kobe Gakuin University for Collaborative Research A.

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