〈Original article〉

Mitochondrial respiratory chain supercomplexes in human mononuclear leukocytes.

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Summary An association of mitochondrial respiratory chain complexes is called a supercomplex. These supercomplexes enhance energy production and reduce electron leaking. Destabilization of supercomplexes may increase oxidative stress and mitochondrial dysfunction, and aging is a trigger for mitochondrial dysfunction. We used high-resolution clear native-polyacrylamide gel electrophoresis (hrCN-PAGE) and two-dimensional hrCN/SDS-PAGE to examine supercomplex formation in human mononuclear leukocytes and estimate complex I proportions for supercomplexes. The results were then compared in relation to sex and age group. Supercomplex clusters were more stable in females than in males. Larger supercomplexes were more stable among subjects aged 55–67 years than in those aged 21–25 years; smaller supercomplexes were less stable among subjects aged 55–67 years than in those aged 21–25 years.

Key words: Mononuclear leukocytes, Mitochondria, Respiratory chain complex, Supercomplex, High resolution clear native electrophoresis

1. Introduction

Mitochondrial respiratory chain complexes are responsible for the oxidative phosphorylation system. Among the various types of associations of respiratory complexes, the respirasome is the unit that performs complete respiration from NADH to oxygen. The structural and functional organization of respirasomes has been examined and explained in the fluid model, solid model, and plasticity model[1]. Associations of mitochondrial respiratory chain complexes are referred to as supercomplexes, which are almost completely quantitatively solubilized by detergents such as dodecyl maltoside, digitonin and Triton X-100 and separated by means of blue native polyacrylamide gel electrophoresis (BN-PAGE) in a functionally active state. Several types of supercomplexes have been detected by BN-PAGE, and each component of the supercomplexes was revealed by two-dimensional BN/SDS-PAGE or BN/BN-PAGE[2]. It has been reported that supercomplex association in respirasomes offers advantages such as enhanced electron transfer and substrate channeling[3]. Although complex I is a major source of reactive oxygen species (ROS)[4], supercomplex association prevents excessive ROS from complex I, because of decreased electron leakage[5].

Age-related decline in respiratory complex

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activity was measured by cuvette assay in rat heart, skeletal muscle[6], brain, and lymphocytes[7]. Complex III activity via complex II was also slightly decreased in human leukocytes[8]. Protein expression of each complex tends to decrease, and alteration of supercomplex formation can be broadly classified into three categories[9-13]: stability may be conserved as in rat gastrocnemius, cortex and human skin, decomposition is enhanced as in rat heart, and alteration is not observed as in rat kidney.

High-resolution clear native (hrCN)-PAGE contains non-colored mixtures of anionic and neutral detergents instead of CBB G-250 dye. These mixed micelles impose a charge shift on membrane proteins, which enhances their anodic migration and improves membrane protein solubility during electrophoresis. Furthermore, non-stained protein has advantages when CBB G-250 interferes with techniques required for further analysis, such as in-gel catalytic activity assay, fluorescence resonance energy transfer (FRET) analysis, or western blotting[14]. The sensitivity of hrCN-PAGE is at least 10 times that of BN-PAGE for in-gel ATP hydrolysis assay[14]. Both BN-PAGE and hrCN-PAGE are effective in detecting supercomplex activity[14-15], since these PAGE techniques avoid nonspecific interference by running those substances out of the gels[16].

In this study, we used hrCN-PAGE and two-dimensional hrCN/SDS-PAGE to examine supercomplex formation in human mononuclear leukocytes and estimate complex I proportions for supercomplexes. The results were then compared in relation to sex and age group.

2. Materials and methods

1. Blood specimens

The study was conducted in compliance with the Declaration of Helsinki and applicable national laws and regulations and was approved by the Ethics Committee of the Faculty of Science, Toho University (#Biology 4 and #Biology 27-1). Written informed consent was obtained from all subjects. Blood specimens were donated by healthy volunteers. The sample comprised 11 males in the age group 21–25 years (mean age ± SD, 21.6 ± 1.2 years), five males in the age group 55–67 years (60.4 ± 4.7), seven females in the age group 21–22 years (21.1 ± 0.4), and four females in the age group 59–63 years (61.5 ± 1.7). Leukocytes were isolated according to the procedure for Polymorphprep (AXIS-SHIELD, Norway). Blood samples were centrifuged at 1,000 × g for 30 min at room temperature (Hi-mac CT6D, Hitachi, Japan). Then, the upper layer of the two leukocyte layers was collected and used as mononuclear leukocytes, which were then washed three times with phosphate-buffered saline (PBS) and stored at −80°C until analysis.

2. Solubilization of membrane proteins

Mononuclear leukocytes were disrupted by using three freeze-thaw cycles with 50 mM potassium phosphate buffer (pH 7.4) and ultracentrifugation at 98,600 × g for 1 h at 4°C (Optima TLX Ultracentrifuge 120,000 rpm, Rotor: TLA45, BECKMAN, USA). The precipitate was washed with potassium phosphate buffer and recollected by ultracentrifugation. The membrane fraction was solubilized by membrane lysis buffer (MLB: 500 mM ε-aminocaproic acid, 50 mM sodium chloride, and 50 mM Bis-Tris, pH 7.0) in the presence of digitonin and n-dodecyl-β-D-maltoside (DDM) and mixed in a tube mixer at maximum speed for 30 min at 4°C (MT-360, TOMY, Japan). The detergent-to-protein ratios (g/g) in MLB were 0.1–1.0 of digitonin and 0.005–0.1 of DDM, respectively. Then the membrane fraction was ultracentrifuged at 98,600 × g for 30 min at 4°C, and the supernatant was collected. Protein concentration was determined by the Bradford method (Bradford protein assay dye reagent concentration, Bio-Rad, USA) using bovine serum albumin as the standard (WAKO, Japan).

3. Electrophoresis and visualization of mitochondrial respiratory chain complexes

Respiratory chain complexes were separated by hrCN-PAGE[14], with minor modifications. Sample buffer contained 50 mM sodium chloride, 10%

glycerol, 0.001% ponceau S, and 0.005% CBB G-250 in 50 mM Bis-Tris (pH 7.0). Two hundred micrograms of the sample was divided and applied to 10 lanes of 3–14% gel for hrCN-PAGE (500 mM ε-aminocaproic acid and 50 mM Bis-Tris, pH 7.0). The gradient gel was made by a gradient former (Model 485, Bio-Rad, USA) with a Mini-PROTEAN 3 multicasting chamber (Bio-Rad, USA). The anode buffer was Native PAGE running buffer (ThermoFisher Scientific, USA), and the cathode buffer contained 0.05% deoxycholic acid and 0.01% DDM in anode buffer. NativeMark (ThermoFisher Scientific, USA) was used as the mobility marker, to indicate apparent molecular mass.

The activity of complex I was determined by the in-gel activity assay method[14], with minor modifications. The hrCN-PAGE gels were incubated with assay buffer (0.1mg/ml NADH and 0.2mg/ml nitro blue tetrazolium chloride in 2 mM Tris-HCl, pH 7.4) at 37°C in darkness (Figs.1, 2A1 and 2B1).

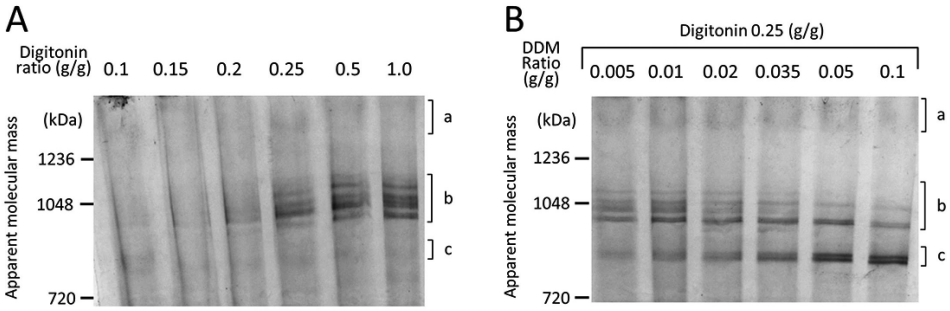

Fig. 1 Solubilization of mononuclear leukocyte membrane.
Mononuclear leukocyte membranes were solubilized by digitonin (A) or digitonin and n-dodecyl-β-D-maltoside (DDM) (B). The activity of complex I was visualized by in-gel activity assay. The detergent-to-protein ratio is shown for each lane. Twenty micrograms of protein was applied in each lane.

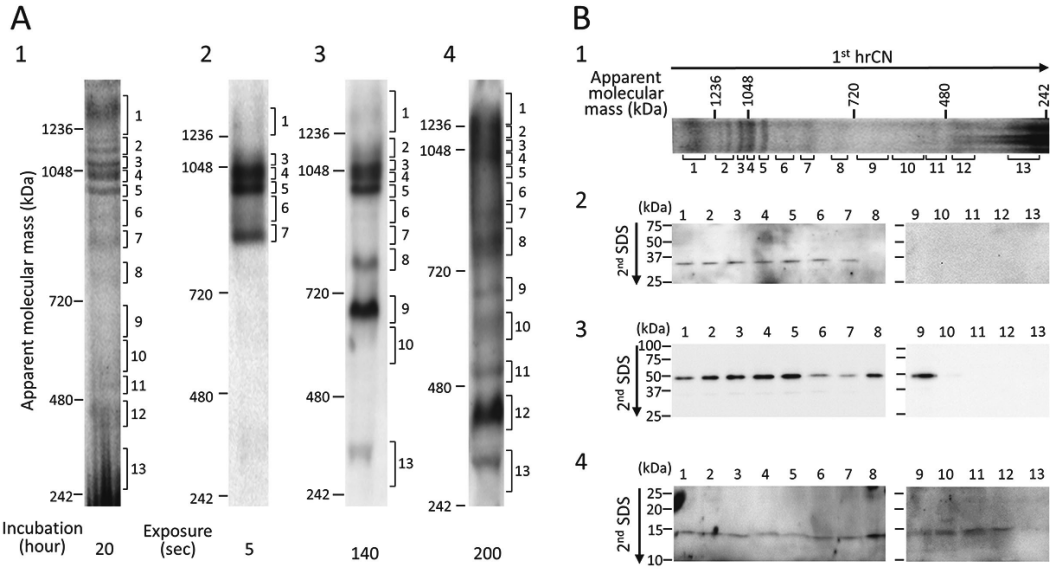

Fig. 2 Components of respiratory supercomplexes.
The components of respiratory supercomplexes were revealed by hrCN-PAGE (A) and two-dimensional hrCN/SDS-PAGE (B). The complex I activity of solubilized mononuclear leukocyte membrane was determined by in-gel activity assay (A1 and B1). Complexes I (A2 and B2), III (A3 and B3), and IV (A4 and B4) were detected by western blotting. The sample (lane) number in Figures B2, B3 and B4 corresponds to the band number in Figures A1–4 and B1.

For two-dimensional hrCN/SDS-PAGE (Fig. 2B) after in-gel activity assay of complex I, each piece labeled "1" to "13" on hrCN-PAGE was cut out, incubated with 2% SDS, 10% 2-mercaptoethanol, 25% glycerol, and 0.01% bromophenol blue in 62.5 mM Tris-HCl (pH 6.8) for 30 min at 37°C, and then applied to SDS-PAGE as the second dimension. For western blotting (Figs.2A2 to 4), hrCN-PAGE gels were incubated for 30 min at 37°C with 5 mM 2-mercaptoethanol, 0.1% SDS, and 192 mM glycine in 25 mM Tris-HCl (pH 8.3). The gels were then transferred onto Immun-Blot PVDF Membrane (Bio-Rad, USA) by using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad, USA). Respiratory complexes I, III, and IV were detected using the following antibodies: 1:5000 dilution anti-NDUFA9 [20C11B11B11] ab14713, 1:7500 dilution anti-Core I [16D10AD9AH5] ab110252, and 1:3000 dilution anti-Cox IV [20E8C12] ab14744 (Abcam, USA), respectively. The second antibody was anti-mouse IgG (H+L) antibody 515-035-062 (Jackson ImmunoResearch, USA). All antibodies were diluted in Can Get Signal Immunoreaction Enhancer Solution (TOYOBO, Japan), and EzWestLumi plus (ATTO, Japan), ImmunoStar Basic (WAKO, Japan), and ECL Select Western Blotting Detection Reagent (GE Healthcare, UK) were used for horseradish peroxidase detection. The signal was detected with a ChemiDoc XRS+ system (Bio-Rad, USA) or ImageQuant LAS 4010 (GE Healthcare, UK). The image of the in-gel activity assay of complex I was detected by using a ChemiDoc XRS+ system, and the intensity of each band labeled "1" to "7" in Figure 2A1 was analyzed using Image Lab ver. 5.1 software (Bio-Rad, USA).

4. Statistical analysis

Analysis of complex I activity from one blood specimen was carried out in triplicate from an independent solubilization of the membrane fraction. The intensity of each band labeled "1" to "7" in Figure 2A1 was summed and considered as 1 after logit transformation, and analyzed using Welch's t-test (Fig. 3). P values of <0.05 and <0.01 were considered to indicate statistical significance[17-19]. All statistical analysis was performed with R version 3.2.2.

3. Results

1. Solubilization of human mononuclear leukocyte membranes

Complex I activity in solubilized mononuclear leukocyte membranes was determined by in-gel activity assay. First, mononuclear leukocyte membranes were solubilized by digitonin, and several bands of active complex I were detected in area "b" (Fig. 1A). Theoretically, the mobility of proteins during hrCN-PAGE is essentially governed by their molecular size, as charge shift molecules bind to a protein surface and mask intrinsic charge

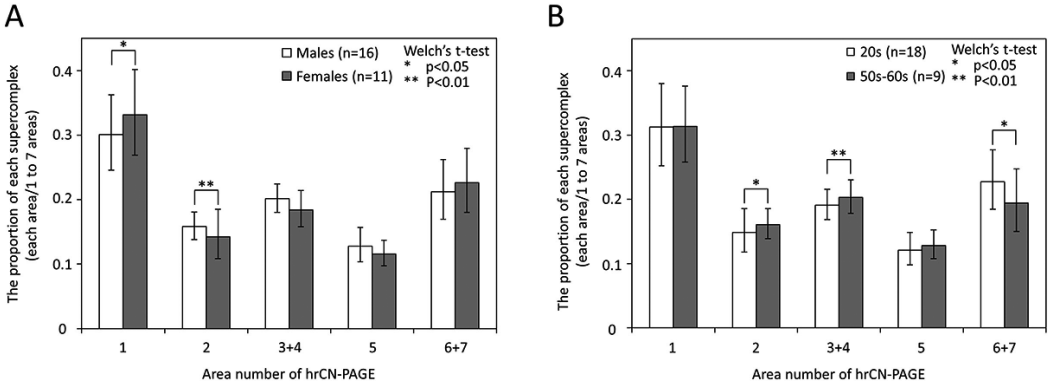

Fig. 3 Proportion of supercomplexes, as estimated from complex I activity.
The proportion of each supercomplex was compared between males and females (A) and between age groups 21–25 years and 55–67 years (B). Intensities of in-gel activity assay of complex I were analyzed using Welch's t-test after logit transformation.

effects[2]. Therefore, the increasing intensities of the smaller molecular bands in area "b" suggest that supercomplexes dissociate in a concentration-dependent manner. Therefore, we chose a digitonin concentration of 0.25 g/g for solubilization of membrane proteins, and further examined if additional DDM had a positive effect on solubilizing membrane proteins, without dissociation of supercomplexes (Fig. 1B). Similarly, the intensities of the bands in area "c" increased in a concentration–dependent manner, while in the case of 0.01 g/g DDM with 0.25 g/g digitonin, the intensity of the areas for supercomplex clusters "a" and supercomplexes "b" were the highest of any DDM concentration. Thus, 0.25 g/g digitonin and 0.01 g/g DDM were added to solubilize the membrane fraction in the following experiment.

2. Supercomplex components

Supercomplexes were separated by hrCN-PAGE, and visualized by in-gel activity assay of complex I (Fig. 2A1) or western blotting using anti-NDUFA9 (Fig. 2A2), anti-Core I (Fig. 2A3), and anti-Cox IV (Fig. 2A4). Although area "2" contained two weak bands that were revealed by complex I activity staining, no bands were detected by western blotting (Figs. 2A1 and 2). Two-dimensional hrCN/SDS-PAGE revealed that areas "1" to "7" had complex I as a component of supercomplexes (Figs. 2B1 and 2B2). Since NDUFA9 is located inside the supercomplex structure[20-21], it is difficult to detect in supercomplexes[22]. Similarly, Core I signals in areas "1" and "2" were weak on hrCN-PAGE but were detected by hrCN/SDS-PAGE (Figs. 2A3 and 2B3). Cox IV signals were detected in areas "1" to "12" by both hrCN-PAGE and hrCN/SDS-PAGE (Figs. 2A4 and 2B4). Although area "13" exhibited bands in Figures A1, 3 and 4, they seemed to be nonspecific, as there was no band in two-dimensional hrCN/SDS-PAGE. These findings suggest that the supercomplexes in areas "1" to "7" had complexes I, III and IV, while areas "8" to "10" had complexes III and IV. Areas "11" to "12" were likely an individual complex IV or complex IV oligomer.

3. Supercomplex proportions, as estimated from complex I activity

Because the boundary separating areas "3" and "4" were unclear, the intensities of these areas were combined. The same method was used for areas "6" and "7". Then, the proportions of intensities for areas "1" to "7" were compared by sex (Fig. 3A) and age group (Fig. 3B). The proportion in area "1" was higher for females than for males ($p < 0.05$), whereas the proportion in area "2" was higher for males ($p < 0.01$). The proportions in areas "2" and "3+4" were higher for age group 55–67 years than for age group 21–25 years ($p < 0.05$ and $p < 0.01$, respectively), whereas the proportion for area "6+7" was higher for age group 21–25 years ($p < 0.05$).

4. Discussion

We detected a variety of supercomplexes by hrCN-PAGE and compared the proportions of supercomplexes in relation to sex and age group. Detection of complex III and IV proteins in complex I active areas "1" to "7" confirmed the findings of Van Raam and colleagues[23]. All complex I proteins in the present analysis were supercomplexes. The coexistence model suggests that the maximum number of components in a supercomplex would be $I_1III_2IV_4$ and that a maximum of six types of $I_1III_nIV_n$ supercomplexes would be detected by BN-PAGE[24]. However, the linear association model suggests that BN-PAGE can separate fragments of linear association as $I_nIII_nIV_n$ supercomplexes[2, 25]. Indeed, many bands of $I_nIII_nIV_n$ supercomplexes were detected by BN-PAGE and in-gel activity assay of complex I and IV in mouse heart mitochondria[22]. Our results support the linear association model and suggest that the supercomplex in area "1" would be the $I_nIII_nIV_n$ supercomplex cluster.

We compared the stability of supercomplexes by sex and age group. Our results agree with those of previous reports, which indicate that complex I protein decreases to a greater extent, as compared with complexes III and IV, and that supercomplex stability is conserved during aging[9-11]. Larger supercomplexes, such as the proportions of areas "2"

and “3+4”, were higher for the age group 55–67 years than for the age group 21–25 years, while the proportions of smaller supercomplexes, such as area “6+7”, were higher for younger adults. Therefore, in human mononuclear leukocytes, supercomplex stability is conserved during aging, and the relative expression of complex I may decrease, as compared with expressions of complexes III and IV. Therefore, stabilization and preservation of supercomplexes may help maintain energy production. Supercomplex clusters such as the proportion of area “1” were more stable in females than in males ($p < 0.05$), while some supercomplexes, such as the proportion of area “2”, were more stable in males ($p < 0.01$). Dissociation of the supercomplex cluster in area “1” may lead to increases in supercomplexes in area “2”. In addition, deterioration of supercomplexes may increase ROS production. Our results indicate that, since supercomplexes are more stable in females than in males, ROS production may be lower, energy production may be higher, and lifespan may be longer in females.

Conflicts of interest

The authors have declared no conflict of interest.

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