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RESEARCH PAPER

Isolation protocols and mitochondrial content for plasma extracellular vesicles

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Abstract

Mitochondrial content has been reported outside of cells either within extracellular vesicles (EVs) or as free mitochondria. Mitochondrial EVs can potentially play multiple physiological and pathophysiological roles. To understand their functions, isolation protocols to separate mitochondrial EVs from other mitochondrial content need to be established. In the present work, we use a multiple reaction monitoring assay with isotope labeled internal standards to quantify 11 mitochondrial, 6 plasma membrane-specific, 4 endosomal membrane-specific, and 2 soluble proteins to evaluate the efficiency of chromatographic isolation of mitochondrial EVs. The isolation protocol includes ultracentrifugation, size exclusion chromatography, and chromatography on immobilized heparin. All protein concentrations were normalized to the concentration of ATP synthase alpha subunit to generate a ratio that allows comparison of different samples obtained during the isolation. We have shown that initial samples after ultracentrifugation are contaminated with non-EV mitochondrial content that cannot be separated from EVs using size exclusion chromatography, but can be efficiently separated from EVs on the column with immobilized heparin.

Keywords Mass spectrometry · Targeted proteomics · Extracellular vesicles · Mitochondria · Heparin-Sepharose

Introduction

Physiological and pathophysiological intercellular mitochondrial transfer has been reported for various cells both in vitro and in vivo [1–9]. There are numerous mechanisms of intercellular mitochondrial transfer [1], including those mediated by extracellular vesicles (EVs). EVs carry proteins and nucleic acids between cells, and a number of reports demonstrated the presence of mitochondrial proteins and mtDNA in EV cargo [1–9]. The functional outcome of intercellular mitochondrial transfer depends on the conditions and the cells involved. Delivery of functional mitochondria aims to restore the recipient cell metabolic activity, thereby improving their survival. However, delivery of damaged mitochondria can activate the innate immune system and enhance inflammation. Another level of complexity arises from the fact that free mitochondria and mitochondrial-derived vesicles (MDVs) have been found in the intercellular space as well [1, 8, 9]. MDVs are 70 to 150 nm in diameter and generated through the budding of damaged or dysfunctional mitochondria. MDVs are typically transported intracellularly to lysosomes and peroxisomes to be degraded [7–9]. However, under stress conditions, MDVs can be packed inside EVs and extracellularly discharged [7, 8]. All of these observations point to a need for separation protocols that can isolate specific subpopulations of EVs and detection methods that can be used to assess the efficiency of isolation of the desired subpopulations prior to studying functional roles of mitochondrial EVs.

Currently used techniques for EV isolation mostly rely on separation by size and/or density [10–12] and poorly applicable to separation of mitochondrial EVs and free mitochondria. There are also reports on immuno-based isolation techniques [11, 12] that include an elution step with glycine buffer at pH 2.2. Unfortunately, acidic elution might alter the

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properties of isolated EVs and make immune-based techniques of limited application.

In the present work, we utilize an affinity chromatography to separate mitochondrial EVs and free mitochondria. We also propose to use a multiple reaction monitoring (MRM) mass spectrometry assay with isotope labeled internal standards [13–15] to quantify several mitochondrial, EVs, and soluble proteins in order to evaluate the efficiency of chromatographic isolation of mitochondrial EVs from human plasma.

Materials and methods

Isolation of EV subpopulations

An aliquot (100 mL) of pooled male human plasma K2EDTA (BioreclamationIVT, Westbury, NY) was centrifuged at $2000 \times g_n$ for 10 min, and the supernatant was subjected to $20{,}000 \times g_n$ centrifugation for 20 min. The pellet was resuspended in 2 mL of PBS and centrifuged again at 20,000 g_n for 20 min. The PBS-washed pellet was called 20 K EVs. The supernatant of 20,000 g_n centrifugation was further centrifuged at $106{,}000 \times g_n$ for 60 min using a Beckman Ti 70 rotor and XL-90 ultracentrifuge. The pellet was dissolved in 1.0 mL of PBS and centrifuged again at $106{,}000 \times g_n$ for 60 min using Beckman TLA-55 rotor and TL-100 ultracentrifuge. The PBS-washed pellet was called 106 K EVs. Both 20 K EVs and 106 K EVs were then loaded on a Superdex 200 Increase 10/300 GL column equilibrated in PBS. For size exclusion chromatography (SEC), an AKTA FPLC (Amersham Biosciences, Piscataway, NJ, USA) was used. The flow rate was 0.4 mL/min, and 0.5 mL fractions were collected. Void volume fractions were pooled to generate 20 K-SEC EVs and 106 K-SEC EVs, respectively. Both of these samples were further separated on the HiTrap Heparin HP (1 mL) column (GE Healthcare) using AKTA FPLC. The column was equilibrated in PBS. Samples in PBS were loaded, and the column was washed with 10 column volumes of PBS. The elution was performed with 0.5 mol/L NaCL in 20 mmol/L phosphate buffer (pH 7.3). The flow rate was 0.4 mL/min, and 0.5 mL fractions were collected.

15 N-Labeled internal standards for quantitative proteomic analysis

Two new ^{15}N-labeled quantitative concatamers (QconCATs) to quantify mitochondrial proteins (Mito1 and Mito2) were generated (Fig. S1, Supplementary Information). The design, expression, purification, and characterization of QconCATs have been previously described in detail [16–18]. All of these QconCATs were designed for quantification of various EV and non-EV proteins. However, not all of them can be quantified in every specific MRM assay. For those proteins that were quantified in this study, three optimal MRM transitions per each Q-peptide are shown in Table S1 (Supplementary Information).

Sample processing and MRM assay

The protein samples in 50 mmol/L NH_4HCO_3 were supplemented with ^{15}N-labeled QconCATs (from 1 to 5 pmol each). The following digestion with trypsin was performed as described before [19]. MRM assays were performed on an Agilent 6490 iFunnel Triple Quadrupole LC/MS system (Santa Clara, CA, USA) equipped with an Agilent 1200 HPLC system (Santa Clara, CA, USA) as described before [19]. All precursors were + 2 charge, and product ions were y-ions with + 1 charge.

Data analysis

Every transition measured per peptide was taken as an individual measurement. MRM peak area integration was performed using Skyline (University of Washington). Peak integration was manually inspected and adjusted, if necessary. The peak ratios from transitions were averaged to yield the peptide ratios. Every transition measurement was taken as a single measurement. We measured 3 transition per peptide and 2 peptides per protein in 2 biological replicates. Therefore, data are represented as the mean ± SD ($n = 12$).

Results and discussion

Internal standards for MRM assay

We have designed, expressed, and purified two new QconCATs (Mito1 and Mito2) that allow quantification of 13 mitochondrial proteins (Fig. S1, Supplementary Information). The selection was based on their high abundance, membrane association, and availability of two or more tryptic peptides per protein suitable for MRM assay. ^{15}N-labeled QconCATs were expressed in *E. coli* with ^{15}N incorporation being higher than 99% based on comparison of labeled and unlabeled peaks for selected peptides in the MRM assay. Purity of QconCATs was estimated to be higher than 95% based on SDS-PAGE. No adjustments for labeling and purity were made during the quantification accordingly. Peptides from two proteins included in the Mito1 and Mito2 were never detected in our experiments. Therefore, only 11 proteins with a

confident quantification are listed below: NADH dehydrogenase [ubiquinone] 1, subunit C2; NADH dehydrogenase [ubiquinone], flavoprotein 1; cytochrome b; ATP synthase, alpha subunit; ATP synthase, beta subunit; NADH dehydrogenase [ubiquinone], iron-sulfur protein 3; cytochrome b-c1 complex, subunit 1; cytochrome b-c1 complex, subunit Rieske; cytochrome c1; ATP synthase F(0) complex, subunit B1; and ATP synthase F(0) complex, subunit C1. It is important to emphasize that all of these proteins can be found strictly in mitochondria and represent established mitochondrial markers.

To quantify plasma membrane-specific proteins, endosomal membrane-specific proteins, and common soluble proteins, six QconCATs that were previously obtained [16–18] were used as well. In total, we focused of quantification of six cellular membrane-specific proteins (integrin alpha-IIb; integrin beta-3; platelet glycoprotein Ib alpha; platelet glycoprotein Ib beta; platelet glycoprotein V; and platelet glycoprotein IX), four endosomal membrane-specific proteins (integrin alpha-2, P-selectin, cytochrome P-450 5A1, and cyclooxygenase 2), and two common soluble proteins (serum albumin and alpha-2-microglobulin). Cellular membrane-specific proteins include several platelet proteins since platelets presumably are the major source of EVs in plasma samples [20].

Isolation of EV subpopulations

We started the isolation with two traditional steps, differential centrifugation and SEC. Differential centrifugation allows the removal of bulk a plasma proteins and separation of the whole pool of EVs into two commonly used fractions (Fig. 1). After removing free cells by 2000 g_n centrifugation, the first fraction (20 K EVs) was pulled down at 20,000 g_n centrifugation of 2 K plasma. 20 K EVs fraction represents large EVs mainly generated by budding of the cellular membrane. If any extracellular mitochondria are present, they should be present in this fraction.

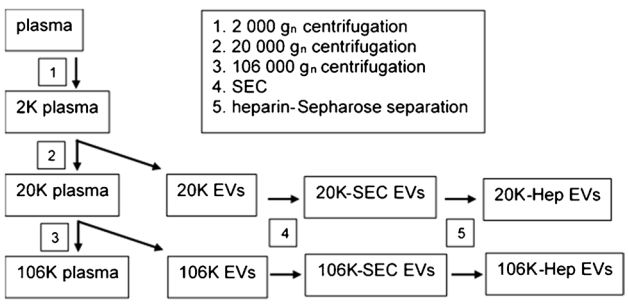

Fig. 1 Scheme of EV sample isolation

The second fraction (106 K EVs) was pulled down by 106,000 g_n centrifugation of 20 K plasma. 106 K EVs fraction represents small EVs that can originate from both cellular and endosomal membranes. This fraction also can carry small free MDVs, if any. SEC of the 20 K EVs and 106 K EVs fractions resulted in 20 K-SEC EVs and 106 K-SEC EVs (Fig. 1) collected as void-volume elution samples. These two samples were further separated on the HiTrap Heparin HP column to obtain 20 K-Hep EVs and 106 K-Hep EVs, respectively. Chromatography on heparin-Sepharose was selected because of reported successful isolation of EVs on immobilized heparin [21] and observation that mitochondria are not retained by heparin-Sepharose [22].

MRM data for selected mitochondrial, cellular-membrane specific, endosomal-membrane specific, and soluble proteins are summarized in Tables 1 and 2 for 20 K and 106 K samples, respectively. The original data in pmol of targeted protein/mg of total proteins are presented as a ratio to the concentration of ATP synthase alpha subunit (highlighted in red). For example, the concentration of ATP synthase alpha subunit was 10 pmol/mg of total protein in 20 K EVs sample (Table 1). Concentrations of all other proteins in this sample were divided by 10 and presented as a ratio normalized to ATP synthase alpha subunit. The same calculations have been done for all other samples keeping in mind that concentrations of ATP synthase alpha subunit were 34 pmol/mg of total protein in 20 K-SEC EVs, 7 pmol/mg of total protein in 20 K-Hep EVs, 1.3 pmol/mg of total protein in 106 K EVs, 3.2 pmol/mg of total protein in 106-SEC EVs, and 0.9 pmol/mg of total protein in 106 K-Hep EVs.

Comparison of these ratios was performed in two different ways. The first was a comparison between different steps of isolation. This analysis provides information about enrichment of a specific group or, in other words, about efficiency of separation of different protein groups. The second was a comparison inside the specific protein group. This analysis can provide information about separation of potential subpopulations inside the group, if any.

We start with a summary of data for 20 K EVs. For soluble proteins, we see an expected significant decrease in ratios after SEC. SEC is indeed an efficient way to separate EVs from the bulk of plasma soluble proteins. Accordingly, the ratios for plasma membrane-specific and endosomal membrane specific proteins increased. However, this increase is small and probably reflects two overlapping processes, partial purification and partial loss of 20 K EVs during SEC. Ratios for mitochondrial proteins basically remain the same pointing to co-elution of EVs and mitochondrial material. In other words, SEC is not

Table 1 Ratios for 20 K EVs samples

Proteins	20 K EVs	20 K-SEC EVs	20 K-Hep EVs
Soluble			
Serum albumin	64.0 ± 12.3	2.2 ± 0.6	8.8 ± 2.9
Alpha-2-macroglobulin	25.5 ± 0.83	0.9 ± 0.2	1.8 ± 0.7
Mitochondrial			
NADH dehydrogenase, subunit C2	0.18 ± 0.05	0.22 ± 0.06	0.17 ± 0.05
NADH dehydrogenase, flavoprotein 1	0.20 ± 0.06	0.16 ± 0.06	0.18 ± 0.06
NADH dehydrogenase, iron-sulfur protein 3	0.15 ± 0.05	0.24 ± 0.06	0.24 ± 0.05
Cytochrome b	0.22 ± 0.06	0.22 ± 0.06	0.17 ± 0.05
Cytochrome c1	0.24 ± 0.06	0.26 ± 0.06	0.24 ± 0.05
Cytochrome b-c1 complex, subunit 1	0.22 ± 0.06	0.21 ± 0.06	0.18 ± 0.04
Cytochrome b-c1 complex, subunit Rieske	0.22 ± 0.05	0.20 ± 0.05	0.22 ± 0.04
ATP synthase, alpha subunit	1.0	1.0	1.0
ATP synthase, beta subunit	1.0 ± 0.04	1.0 ± 0.04	1.0 ± 0.06
ATP synthase F(0) complex, subunit B1	0.03 ± 0.01	0.04 ± 0.01	0.03 ± 0.01
ATP synthase F(0) complex, subunit C1	0.19 ± 0.05	0.16 ± 0.05	0.16 ± 0.03
Plasma membrane-specific			
Integrin alpha-IIb	7.9 ± 2.4	8.9 ± 3.0	34.5 ± 10.1
Integrin beta-3	7.2 ± 2.5	8.3 ± 2.7	31.8 ± 10.4
Platelet glycoprotein Ib alpha	1.1 ± 0.4	1.3 ± 0.3	5.0 ± 1.7
Platelet glycoprotein Ib beta	1.1 ± 0.3	1.5 ± 0.3	5.0 ± 1.7
Platelet glycoprotein V	1.1 ± 0.3	1.2 ± 0.3	5.0 ± 1.9
Platelet glycoprotein IX	1.0 ± 0.2	1.1 ± 0.3	4.7 ± 1.7
Endosomal membrane-specific			
Integrin alpha-2	3.0 ± 0.8	1.7 ± 0.05	2.9 ± 0.7
P-selectin	8.3 ± 2.2	4.62 ± 0.19	7.9 ± 2.4
Cytochrome P-450 5A1,	0.3 ± 0.1	0.15 ± 0.06	0.26 ± 0.06
Cyclooxygenase 2	0.5 ± 0.1	0.29 ± 0.10	0.49 ± 0.12

MRM measurements were performed in duplicate for three transitions per peptide and two peptides per protein ($n = 12$). Obtained protein concentrations in pmol/mg of total protein were normalized to the concentration of ATP synthase alpha subunit to generate a ratio for sample comparison. Data are presented as the mean ± SD

efficient for their separation. After heparin-Sepharose, the major observation is that the ratios for plasma membrane-specific proteins strongly increased while the ratios for mitochondrial proteins remain unchanged. A similar trend was observed for endosomal membrane-specific proteins, but on a lower scale reflecting a small amount of endosomal membrane-specific EVs present in the total 20 K EVs. Taken together, we have a clear sign that chromatography on heparin-Sepharose results in the removal of mitochondrial material from 20 K EVs. In summary, our results point to the efficient separation of mitochondrial EVs from non-EV mitochondrial material that is probably represented by free extracellular mitochondria.

Comparison of the ratios inside mitochondrial, plasma membrane-specific, and endosomal membrane-specific protein groups in various fractions of 20 K EVs (Table 1) shows small variations, although overall it seems that SEC and chromatography on immobilized heparin does not change the ratios inside these protein groups. It means that we do not see signs of subpopulations present or separated. This conclusion has a caveat because for each group, we only quantified representative proteins. For example, mitochondrial proteins in this study represent the mitochondrial respiratory chain and may not necessarily reflect potential existence of various MDVs. For plasma membrane-specific and endosomal membrane specific proteins, there is the same limitation that our experimental approach might not be inclusive enough to resolve potentially existing subpopulations.

Data for 106 K EVs shows the presence of mitochondrial proteins (Table 2), which is an important observation since 20 K plasma (Fig. 1) is expected to be devoid of

Table 2 Ratios for 106 K EVs samples

Proteins	106K EVs	106K-SEC EVs	106K-Hep EVs
Soluble			
Serum albumin	262 ± 74	12.5 ± 3.4	11.6 ± 4.4
Alpha-2-macroglobulin	123 ± 33	3.9 ± 1.5	2.3 ± 0.8
Mitochondrial			
NADH dehydrogenase, subunit C2	0.22 ± 0.06	ND	ND
NADH dehydrogenase, flavoprotein 1	0.16 ± 0.05	0.16 ± 0.05	0.18 ± 0.05
NADH dehydrogenase, iron-sulfur protein 3	0.15 ± 0.05	0.17 ± 0.05	0.17 ± 0.05
Cytochrome b	0.16 ± 0.05	0.21 ± 0.06	ND
Cytochrome c1	0.14 ± 0.05	0.19 ± 0.05	ND
Cytochrome b-c1 complex, subunit 1	0.18 ± 0.05	0.19 ± 0.05	ND
Cytochrome b-c1 complex, subunit Rieske	0.20 ± 0.06	ND	ND
ATP synthase, alpha subunit	1.0	1.0	1.0
ATP synthase, beta subunit	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1
ATP synthase F(0) complex, subunit B1	ND	ND	ND
ATP synthase F(0) complex, subunit C1	0.20 ± 0.04	0.18 ± 0.04	ND
Plasma membrane-specific			
Integrin alpha-IIb	6.9 ± 2.1	9.4 ± 3.0	9.4 ± 3.3
Integrin beta-3	6.2 ± 2.7	8.4 ± 3.3	8.4 ± 3.3
Platelet glycoprotein Ib alpha	0.8 ± 0.2	1.3 ± 0.3	ND
Platelet glycoprotein Ib beta	0.8 ± 0.2	1.1 ± 0.2	ND
Platelet glycoprotein V	0.7 ± 0.2	1.1 ± 0.3	ND
Platelet glycoprotein IX	0.7 ± 0.2	1.1 ± 0.3	0.9 ± 0.2
Endosomal membrane-specific			
Integrin alpha-2	34.6 ± 8.9	47.8 ± 14.3	33.5 ± 9.9
P-selectin	0.7 ± 0.2	18.1 ± 5.5	12.7 ± 4.1
Cytochrome P-450 5A1,	4.6 ± 1.7	6.3 ± 2.5	ND
Cyclooxygenase 2	5.4 ± 2.4	7.2 ± 3.0	5.0 ± 1.8

MRM measurements were performed in duplicate for three transitions per peptide and two peptides per protein ($n = 12$). Obtained protein concentrations in pmol/mg of total protein were normalized to the concentration of ATP synthase alpha subunit to generate a ratio for sample comparison. Data are presented as the mean ± SD

any free mitochondrial material. However, one can also assume the presence of small MDVs that stay soluble after 20,000 g_n centrifugation could contribute to the mitochondrial material detected. Our measurements do not show a difference in ratios of mitochondrial and plasma membrane-specific proteins before and after chromatography on heparin-Sepharose. This is likely because 106 K EVs do not have non-EVs mitochondrial material and all of the mitochondrial proteins detected are entrapped inside 106 K EVs. We observed some decrease in ratios for endosomal membrane-specific proteins likely due to a partial loss of endosomal membrane-specific EVs during the chromatography. Overall, considering the efficiency of chromatography on heparin-Sepharose for isolation of 20 K EVs, we concluded that 106 K EVs do not carry mitochondrial material other than that inside EVs.

Conclusion

Traditional EV isolation approaches, such as differential centrifugation and SEC, are not efficient in separating EVs and free mitochondria or free MDVs. We report here that chromatography on heparin-Sepharose can be helpful in achieving this goal.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00216-022-04465-x.

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Author contribution I.V. Turko designed the research, isolated EVs, and performed MRM experiments. A. Nguyen expressed and purified QconCATs. I.V. Turko and A. Nguyen analyzed results and wrote the paper.

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Declarations

Source of biological material Human plasma K2EDTA was purchased from BioreclamationIVT, Westbury, NY.

Conflict of interest The authors declare no competing interests.

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