Unlocking the Power of Mitochondrial Function: Real-time Assessment of Mitochondrial ATP contents and Response Against Inhibiting and Stimulating Substrates (MitoRAISE)

Eun Sol Chang1,2, Kyoung Song3, Ji-Young Song2, Minjung Sung2, Mi-Sook Lee2, Jung Han Oh1, Yeon Hee Park3, Ji-Yeon Kim3, Kyungsoo Jung3, and Yoon-La Choi1

1Samsung Advanced Institute for Health Sciences & Technology
2Duksung Women’s University
3Samsung Medical Center

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Abstract

Purpose The objective is to develop a real-time Mitochondrial ATP contents and Response Against Inhibiting and Stimulating Substrates (MitoRAISE) assay and assess its potential to evaluate the mitochondrial oxidative phosphorylation function.

Methods MitoRAISE measures the total ATP contents, ATP synthesis capacity, and the response to inhibitory substrates. The measurements were all quantified with an ATP standard curve and validated with in vitro testing. MitoRAISE was then applied using peripheral blood mononuclear cells (PBMCs) from 35 healthy volunteers and 20 breast cancer patients. Result Results from isolated mitochondria, cells with different permeabilization percentages, and cells with damaged mitochondria demonstrated the ability of capturing ATP signals specifically from the mitochondria. Breast cancer PBMCs exhibited a significant increase in glutamic acid- and malic acid-induced mitochondrial ATP synthesis capacity yet a significant decrease in mitochondrial DNA copy number (mtDNA CN) compared to healthy PBMCs. Furthermore, breast cancer PBMCs mostly showed negative correlation between mtDNA CN and parameters from MitoRAISE but healthy individuals showed positive correlation. Conclusion We developed a quick and easy method to detect real-time mitochondrial activity in live cells. Monitoring mitochondrial ATP synthesis capacity and sensitivity to inhibitory substrates could aid in assessing the functional status of mitochondrial oxidative phosphorylation.

Biotech Method

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1Department of Health Sciences and Technology, SAIHST, Sungkyunkwan University School of Medicine, Seoul, South Korea
2Laboratory of Molecular Pathology and Theranostics, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, South Korea
3College of Pharmacy, Duksung Women’s University, Seoul, South Korea
4Division of Hematology-Oncology, Department of Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, South Korea
Corresponding Author:
Kyungsoo Jung, Ph.D.
Laboratory of Molecular Pathology and Theranostics, Samsung Medical Center, Sungkyunkwan University School of Medicine, Irwon-ro 81, Gangnam-go, Seoul 06351, South Korea
E-mail: jks850820@naver.com
Yoon-La Choi, M.D., Ph.D.
Department of Pathology and Translational Genomics,
Samsung Medical Center, Sungkyunkwan University School of Medicine, Irwon-ro 81, Gangnam-go, Seoul 06351, South Korea
E-mail: ylachoi@skku.edu

Running title
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Keywords
Mitochondrial ATP synthesis capacity, mitochondrial function, ATP, mtDNA CN

Abbreviations
ATP: adenosine triphosphate
ADP: adenosine diphosphate
Ap5A: P1, P5-Di (adenosine-5') pentaphosphate pentasodium salt
CCCP: 2-[2-(3-Chlorophenyl)hydrazinylidene]propanedinitrile
DNA: deoxyribonucleic acid
OXPHOS: oxidative phosphorylation
PMP: plasma membrane permeabilizer
PBMC: peripheral blood mononuclear cells
GM: glutamic acid and malic acid
S: succinate
NADH: nicotinamide adenine dinucleotide
FADH_2: flavin adenine dinucleotide
FCCP: carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone

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Conflicts of interest
The authors have no conflict of interest to disclose.

Abstract

Purpose

The objective is to develop a real-time mitochondrial ATP contents and response against inhibiting and stimulating Substrates (MitoRAISE) assay and assess its potential to evaluate the mitochondrial oxidative phosphorylation function.

Methods

MitoRAISE measures the total ATP contents, ATP synthesis capacity, and the response to inhibitory substrates. The measurements were all quantified with an ATP standard curve and validated with in vitro testing. MitoRAISE was then applied using peripheral blood mononuclear cells (PBMCs) from 35 healthy volunteers and 20 breast cancer patients.

Result

Results from isolated mitochondria, cells with different permeabilization percentages, and cells with damaged mitochondria demonstrated the ability of capturing ATP signals specifically from the mitochondria. Breast cancer PBMCs exhibited a significant increase in glutamic acid- and malic acid-induced mitochondrial ATP synthesis capacity yet a significant decrease in mitochondrial DNA copy number (mtDNA CN) compared to healthy PBMCs. Furthermore, breast cancer PBMCs mostly showed negative correlation between mtDNA CN and parameters from MitoRAISE but healthy individuals showed positive correlation.

Conclusion

We developed a quick and easy method to detect real-time mitochondrial activity in live cells. Monitoring mitochondrial ATP synthesis capacity and sensitivity to inhibitory substrates could aid in assessing the functional status of mitochondrial oxidative phosphorylation.

1. Introduction

Mitochondria control the biological processes occurring in almost all cells of the human body, such as the synthesis of iron-sulfur clusters, apoptosis, cell signaling, maintenance of calcium homeostasis, and maintaining reactive oxygen species levels.[1, 2] Above all, mitochondria are renowned for synthesizing high energy phosphate bonds in the form of ATP during oxidative phosphorylation (OXPHOS). During the series of oxidation and reduction reactions within the mitochondrial complexes, electrons follow one of the two major electron transport pathways to finally reduce oxygen to water. One pathway is through complexes I, III, and IV using electrons from NADH, and the other is through complexes II, III, and IV using FADH$_2$ from succinic acid.[3] The continuous pumping of protons increases the hydrogen ion concentration and reduces pH, eventually generating a membrane potential across the inner mitochondrial membrane. This membrane potential is utilized by complex V (ATP synthase) to generate ATP.[4-6]

The significance of OXPHOS function can again be emphasized by the fact that mitochondrial diseases arise from dysfunctional OXPHOS systems.[7] Defective complex I was found to cause inflammation and cell death.[8] Despite the significant influence of OXPHOS in many diseases, its function in cancer has been overlooked because it was previously thought that cancer cells mostly utilize aerobic glycolysis as their main energy-synthesizing process.[9] However, growing evidence has shown that OXPHOS function is upregulated in lung cancer,[10, 11] estrogen receptor (ER)-positive breast cancer,[12] pancreatic cancers,[13] Hodgkin’s lymphoma,[14] and other cancers.[15] In addition, blocking mitochondrial respiration resulted in suppressed tumor growth in xenograft models,[16] further proving that mitochondrial function is essential for tumor cell proliferation. Mitochondrial ATP synthesis function through the OXPHOS system can no longer be overlooked, even in cancer, for it can be representative of the mitochondrial robustness.

Despite the apparent significance of the mitochondrial OXPHOS system and ATP, there is not yet a single assay to measure the mitochondrial function related to ATP production. This study was thus designed to
propose a new method for an accurate, direct, and real-time detection of mitochondrial function through sensitivity to activators and inhibitors in isolated mitochondria, cell lines, and peripheral blood mononuclear cells (PBMCs).

2. Materials and methods

2.1 Isolation of mitochondria from cell lines

Mitochondrial isolation was performed following a previously described protocol.\cite{17} Briefly, cells from two 150-mm dishes were prepared in a homogenizing buffer. Subtilisin A solution (200 μL, 4 mg/mL) was added to the buffer, and the solution was drawn into a 22G syringe 20 times while being incubated in ice. The solution was then centrifuged at 9,000 \( \times g \) for 10 min at 4 °C. The pellet was collected in 500 μL ice-cold respiration buffer, and used for measuring ATP contents and MitoRAISE using the ATPlite Luminescence Assay System (PerkinElmer, Waltham, MA, USA). The isolated mitochondria was quantified using a Pierce BCA protein assay kit (Thermofisher Scientific, Waltham, MA, USA).

2.2 Permeabilization of cellular plasma membrane

Cells or PBMCs were treated with 5–10 nM plasma membrane permeabilizer (PMP) and incubated at room temperature for 10 min. Membrane permeabilization percentage was measured with the percentage of trypan blue stained cells using a Countess automated cell counter (Thermofisher Scientific, Waltham, MA, USA). The acceptable permeabilization rate was 90% or higher in the PMP-treated cells.

2.2 Mitochondrial ATP contents and Response Against Inhibiting and Stimulating Substrates (MitoRAISE)

Cells or PBMCs were seeded into a 96-well white plate along with ATP standard material in 1/2 serial dilutions to construct a standard curve. Up to 50 μL mitochondrial assay solution (MAS) buffer containing 250 μM Ap5A was injected and placed on a plate shaker for 2 min. Next, 50 μL of ATPlite solution with 100 μM ADP was added and the mixture was again placed on a plate shaker for 2 min. After incubation for 10 min, luminescence signals were read five times at 2-minute intervals; 10 μL of activating substrates (10 mM glutamic acid mixed with 10 mM malic acid for complex I and 100 mM succinate for complex II) were added to the wells, and the plate was read five times at 2-minute intervals. Finally, 10 μL of inhibiting substrates (40 μM rotenone for complex I and 800 μM malonate for complex II) were injected into the wells, and the plate was read five times at 2-minute intervals. The values of the samples were adjusted against the ATP standard curve, and we defined the ATP synthesis capacity as the slope of increasing ATP luminescence signal. The reduction of the slope after injection of the inhibitory substrates was defined as the sensitivity to inhibitory substrates.

2.4 Determination of mitochondrial DNA copy number

The mitochondrial DNA copy number (mtDNA CN) was calculated through real-time quantitative polymerase chain reaction (qPCR) using the PRISM® 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The primer sequences (Cosmogenetech, Daejeon, Korea) used are described in supplementary methods. The mtDNA CN was calculated with adjustments to the standard curve of a calibrator, which contained exactly one copy of mtDNA and one copy of nuclear DNA.

2.5 Statistical analyses

Data were processed and figures were generated using RStudio (https://www.rstudio.com), Microsoft Excel, and GraphPad Prism version 9.5.1 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com).

3. Results

3.1 Mitochondrial ATP contents and Response Against Inhibiting and Stimulating Substrates (MitoRAISE) assay development
MitoRAISE is a quick and efficient workflow for the direct and simultaneous measurement of ATP contents, ATP synthesis capacity, and response to inhibitory substrates in less than 2 hours. Figure 1A describes the overall workflow. Cells are permeabilized, treated with adenylate kinase and go through basal reading. The basal luminescence represents the amount of ATP in the sample at the time of measurement. MitoRAISE can simultaneously measures the rate at which ATP is produced in real time after treatment of various activating substrates of the ATP synthesis process. ATP synthesis capacity is expected to reflect the status of mitochondria more accurately because it represents the efficiency of the mitochondrial function. The increasing signal after the injection of activating substrates was labeled ATP synthesis capacity. After activation, inhibitory substrates are added, and the mitochondrial sensitivity to inhibitory substance is calculated as the subtraction of inhibitory slope from the activation slope. Finding the sensitivity to inhibitory substance can further illustrate the ability of the mitochondrial function as it will show how much the inhibitory substance can block mitochondrial ATP synthesis capacity. This would hint the amount of which the pathway related to the inhibitory substrate has been damaged.

3.2 Development of MitoRAISE using isolated mitochondria and viable cells

We first performed the MitoRAISE on isolated mitochondria of A549 cells (Figure 1B). The concentration of the mitochondria was measured through BCA protein quantification. As expected, the ATP synthesis capacity of isolated mitochondria showed correlation with the concentration of the isolated mitochondria. With the doubling of concentration of isolated mitochondria from 2 μg to 4 μg, the ATP synthesis capacity induced by the activators, glutamic acid and malic acid (GM) and succinate (S), also doubled. The addition of GM or S to the mitochondrial assay solution (MAS) buffer with 0 μg mitochondria showed no reaction. We next sought to measure the mitochondrial ATP synthesis capacity from viable cells. To overcome the challenge of inducing the entry of substrates into the cells, we utilized the plasma membrane permeabilizer (PMP). PMP can efficiently induce cellular membrane permeabilization while causing limited mitochondrial damage. 5 nM of PMP was sufficient to fully (100%) permeabilize 1*10^4 cells (Figure 1C, Supporting Figure 1A). To test the importance of permeabilization on finding the full ATP synthesis capacity of cells, we intentionally mixed permeabilized cells with intact cells to obtain <10%, 25%, 40%, and 100% permeabilized populations (Supporting Figure 1B). There was a permeabilization percentage dependent increase in ATP synthesis capacity (Figure 1D). This means that cellular permeability for the substrate to enter the cells is important for ATP measurement.

3.3 Validating MitoRAISE in cell lines

As mentioned before, three different parameters can be tested through a single assay of MitoRAISE: ATP contents, ATP synthesis capacity, and inhibitor sensitivity. We tested the three parameters on A549 cells at different cell counts. We were able to see a cell count dependent increase in ATP signals in both MitoRAISE and commercial ATP kit, in which MitoRAISE was able to sensitively reflect the number of cells through ATP contents in more than 1*10^4 cells (Figure 2A, B). ATP synthesis capacity was measured through MitoRAISE simultaneously, and its results also showed correlation with the cell count from 0.5*10^4 cells (Figure 2B). After the implementation of MitoRAISE and validation from normal cells, it was reexamined on damaged cells. A549 rho-0 cells, with depleted mitochondrial DNA confirmed through gel electrophoresis and qPCR, showed less total ATP contents, no ATP synthesis capacity, and naturally, no response to inhibitors (Supporting Figure 2A-B, Figure 2A-C). A549 cells with damaged mitochondrial membrane potential using either 50 μM 2-[2-(3-Chlorophenyl)hydrazinylidene]propanedinitrile (CCCP) or 50 μM carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) was also tested. When treated with CCCP or FCCP, the mitochondrial membrane potential dissipates as seen through the loss of mitochondrial staining in fluorescence microscopy, and no ATP is predicted to be synthesized (Supporting Figure 2C-D). In accordance with the prediction, no ATP was synthesized and no response to inhibitors was found (Figure 2B-C).

We applied MitoRAISE assay to ten different cell lines originating from various organs. They were screened for their entire mitochondrial function through testing the total ATP contents, ATP synthesis capacity, sensitivity to inhibitory substrates, and mitochondrial DNA copy number (mtDNA CN) (Figure 2D-F, Supporting Figure 3). Several positive correlations were found among the parameters: ATP contents and GM-induced
ATP synthesis capacity ($\rho=0.62$), ATP contents and S-induced ATP synthesis capacity ($\rho=0.76$), ATP contents and mtDNA copy number ($\rho=0.73$). Just as we expected, there was a positive correlation between ATP contents, ATP synthesis capacity, inhibitor sensitivity, and these values were in correlation with mitochondrial DNA copy number.

3.5 In vitro testing results show the possible role of utilizing MitoRAISE in detecting individual’s mitochondrial conditions

To obtain clinical meaning from MitoRAISE data, PBMCs from healthy volunteers and cancer patients were obtained, and the six components of mitochondrial function (GM-induced and S-induced ATP synthesis capacity, rotenone and malonate sensitivity, total ATP contents, and mtDNA CN) were measured (Figures 3A-F). Healthy blood was obtained from both males and females, but breast cancer patients were all females. Interestingly, when compared to healthy females, breast cancer patients had significantly high GM-induced ATP synthesis capacity ($p = 0.006$) while significantly low mtDNA CN ($p<0.001$). When compared to healthy males, breast cancer patients had significantly lower sensitivity to malonate ($p=0.04$) and significantly lower mtDNA copy number ($p=0.001$). When comparing healthy male and female, females had significantly lower GM-induced and S-induced ATP synthesis capacity ($p= 0.048$ and $p=0.019$).

Interestingly, when analyzing the correlation between the six components of mitochondrial function measured through MitoRAISE and some clinical features, we found a moderate to high negative correlation between weight and ATP synthesis capacity and weight and inhibitor sensitivity in both healthy males and females ($\rho = -0.2$–$-0.5$) yet only a weak negative correlation in breast cancer patients ($\rho =-0.08$–$-0.23$). There was a positive correlation between mtDNA CN and ATP synthesis capacity and mtDNA CN and inhibitory substrate sensitivity in healthy females but a negative correlation in breast cancer patients (Figure 3H-J).

Discussion

Since the discovery of ATP in 1929 and its function as an energy carrier in the late 1930s,[18] the importance of synthesizing ATP through OXPHOS system has never been questioned.[19] Among the co-working protein complexes in the mitochondria, mitochondrial complex I is the largest, and its dysfunction is directly linked to mitochondrial malfunction in dementia and cancer.[20, 21] Mitochondrial complex II, on the other hand, is the smallest protein complex involved in both the tricarboxylic acid cycle and OXPHOS. Complex II complications can lead to cancer,[22] cell death, and necrosis.[23]

Currently, assays are available to only estimate the rate of ATP production. Agilent Seahorse XF Analyzer is the most widely used platform for estimating the total ATP produced through calculating oxygen consumption and extracellular acidification rate.[24, 25] Oroboros Oxygraph-O2k also measures mitochondrial respiration to estimate ATP production.[26] Oxygen consumption and ATP synthesis are not directly associated and may be masked by other factors, such as mitochondrial concentration or ATP concentration.[27]

Since measuring oxygen consumption rate may not be an accurate measure of ATP production, there is a need to investigate the actual energy metabolism.[28]

Our assay focuses on measuring the actual change of ATP in the mitochondria in response to activating or inhibiting substrates. Using an agent nontoxic to mitochondria, we were able to permeabilize the cells and directly deliver mitochondrial complex-activating substrates. The protocol can be applied to both adhesion and suspension cells and showed high reproducibility in both cell types. During the development process, the A549 cell line was tested on different dates, yet the GM-induced ATP synthesis capacity was consistently $160 \pm 20$ nm/min, whereas the S-induced ATP synthesis capacity was always $294 \pm 70$ nM/min, which is an error range of only 10%–20% (data not shown). Notably, we were also able to obtain reproducible data from PBMCs. The samples tested immediately after blood collection and after 6 hours of incubation at room temperature only had a 10–20% error range (data not shown).

In addition to the novel measurement of mitochondrial ATP synthesis capacity, MitoRAISE can measure the ATP contents and sensitivity to inhibiting substrates. Finding the sensitivity to inhibiting substrates can be helpful in quickly finding the sensitivity of a patient to a specific drug. For example, when treating a patient
with a drug with known inhibitory effects on the mitochondria, testing the patient PBMCs with MitoRAISE would be a non-invasive method to screen the drug. One of the greatest advantages of MitoRAISE is that it is not limited to just one specific type of activating substrates or inhibiting substrates. We utilized GM and S to activate mitochondrial complexes I and II, but other activating substrates can be utilized. The ATP synthesis capacity can also signify the mitochondrial sensitivity or reactivity to specific substrates. As such, we can utilize MitoRAISE to measure the mitochondrial reactivity to inhibitory substrates.

PBMCs are readily available sources of patient samples and are currently receiving attention for their potential use as predictive risk biomarkers for multiple diseases. Myriad of studies have shown the use of PBMCs as potential tools to determine the inflammatory and metabolic status in various disease states, including chronic fatigue syndrome, and type 2 diabetes. Animal model studies have also shown that the metabolic responses of PBMCs can correlate with tissues such as the liver and brain. Data from our preliminary clinical study showed disease-linked disparity in PBMC mitochondrial activity parameters, in which breast cancer patients showed higher ATP synthesis capacity yet lower mtDNA copy number and lower sensitivity to inhibiting substrates. Also, healthy females showed positive correlation between mitochondrial DNA copy number and ATP synthesis capacity and inhibitory substrate sensitivity meaning more mitochondria meant more ATP synthesis capacity and more reaction to the inhibitory substrates, but breast cancer patients showed slightly negative correlation. When testing the newly developed assay for ATP synthesis capacity, we found differences in the mitochondrial function between gender and between diseased and healthy. It has been shown that upregulated mitochondrial complex I is required for high metastatic features of colorectal cancer cells. Likewise, the upregulated ATP producing capacity in the PBMCs of breast cancer patients could signify the high need of energy in cancer cells. Thus, this newly developed MitoRAISE assay can be a prospective tool in determining mitochondrial function of patients.

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Figures were created with BioRender.com

Author Contribution


References


Figure 1. Development of MitoRAISE protocol

A) Overall workflow of the mitochondrial ATP synthesis capacity protocol. Samples, ranging from cell lines to patient PBMCs were harvested in MAS buffer and permeabilized. After the cells were seeded into a white 96-well plate and treated with the appropriate Ap5A, ADP, and luminescence, the plate was subjected to reading. B) ATP synthesis capacity in isolated mitochondria. Left) Dot plot showing the real time accumulation of ATP signals from 4 μg mitochondria (circle) and MAS buffer with 0 μg mitochondria (triangle) activated by GM and S. Arrows indicate the time in which activating or inhibiting substrates were injected. Right) Bar graph depicting the slope of ATP synthesis capacity after injection of activating substrates, GM and S, to 0 μg, 2 μg, and 4 μg mitochondria. C) Bar graph depicting the slope of ATP synthesis capacity in samples treated with 0 nM, 2 nM, 5 nM, and 10nM PMP in 10,000 cells. D) Bar graph depicting the slope of ATP synthesis capacity in samples with <10%, 25%, 40%, and 100% of the 10,000 cells permeabilized.
Figure 2. MitoRAISE validation in cell lines

A) Total ATP contents of A549 cells at increasing cell count and A549 rho-0 cells found through commercial kit compared with the ATP contents measured through MitoRAISE assay. B) GM-induced and S-induced ATP synthesis capacity of A549 cells at different cell count, A549 rho-0 cells, and A549 cells treated with CCCP or FCCP. C) Correlation between mitochondrial parameters in cell lines.

GM: glutamic acid and malic acid, S: succinate, PMP: plasma membrane permeabilizer

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CCCP: 2-[2-(3-Chlorophenyl)hydrazinylidene]propanedinitrile, FCCP: carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone, GM: glutamic acid and malic acid, S: succinate
Figure 3. MitoRAISE in PBMC samples (n=35)

(A–F) Comparison of GM-induced ATP synthesis capacity, S-induced ATP synthesis capacity, ATP contents, rotenone sensitivity, malonate sensitivity, and mtDNA CN among healthy females (n=23), males (n=12), and female breast cancer patients (n=20). (G–I) Generic correlation between factors in male healthy volunteers, female healthy volunteers, and patients with breast cancer. *p ≤ 0.05 **p ≤ 0.01 ****p ≤ 0.001