Fluorescence imaging-based analysis of the mitochondrial permeability transition pore opening in cardiomyocyte-derived H9c2 cells

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Abstract

Mitochondrial function is the key factor in the regulation of energy metabolism and is closely related to the generation and prevention of lifestyle-related diseases. We established a method to observe the opening of the mitochondrial permeability transition pore using fluorescence microscopy and permeabilized cardiomyocyte-derived H9c2 cells. After calcein loading and permeabilization of the cell membrane with digitonin, the cellular calcein fluorescence coincided with that of the mitochondrial fluorescent probe tetramethylrhodamine methylester. Elevation of extracellular Ca2+ concentration, which was accompanied by an increase in intramitochondrial Ca2+ concentration, induced a decrease in calcein fluorescence intensity. This decrease was inhibited by cyclosporine A, an inhibitor of mitochondrial permeability transition pore. Further, the decrease was inhibited by ruthenium red, an inhibitor of the mitochondrial uniporter, and enhanced by diltiazem, which inhibits the mitochondrial Na+-Ca2+ exchanger. The present imaging method would be useful for further studies on the regulation of mitochondrial function under physiological and various pathological conditions related to lifestyle-related diseases.

1. Introduction

Mitochondrial function is the key factor in the regulation of energy metabolism and is closely related to the generation and prevention of lifestyle-related diseases[1-6]. Cardiac muscle, as well as red type skeletal muscle, largely relies on the mitochondrial oxidative phosphorylation as a source of cellular ATP. Loss of mitochondrial function plays a significant role in the pathophysiology of ischemic diseases including angina pectoris and myocardial infarction[7,8]. Especially, cytoplasmic Ca2+ overload during ischemia is one of the major triggers for irreversible cell injury which causes mitochondrial Ca2+ overload and the subsequent decline in mitochondrial function[9]. One of the major events which lead to loss of mitochondrial function is the opening of the mitochondrial permeability transition pore (mPTP). Permeability transition reflects the formation of a nonspecific high-conductance channel in the inner mitochondrial membrane[10-12]. This results in the collapse of the electrochemical gradient of protons across the mitochondrial inner membrane, mitochondrial swelling, and induction of ATP hydrolysis by the FoF1 ATPase. Thus, to study mitochondrial function under various pathological conditions, monitoring or the mPTP opening is indispensable.

In the present study, we intended to establish a fluorescence imaging-based method for the evaluation of mPTP opening in cardiomyocyte-derived H9c2 cells, and to pharmacologically analyze the correlation between mitochondrial Ca2+ dynamics and mPTP status.

2. Methods

H9c2 cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) high glucose (GIBCO) under
humidified 5% CO₂ atmosphere. For the measurement of mPTP opening, H9c2 cardiomyocytes were incubated with calcein (0.5 μM calcein/AM for 15 min at 37°C). The plasmalemmal membrane was permeabilized by perfusion with digitonin (20 μg/ml) in a Ca²⁺-free extramitochondrial solution containing (in mM) 50 KCl, 80 potassium aspartate, 4 sodium pyruvate, 20 HEPES, 3 MgCl₂, 3 Na₂ATP, 5.8 glucose, and 3 EGTA (pH 7.3 with KOH). The quenching of cytosolic calcein was achieved by addition of 1 mmol/L CoCl₂ to the solution. On measurement of mPTP opening, the Ca²⁺ concentration of the extramitochondrial solution was changed from 0 nM to 300 nM. The free Ca²⁺ concentration was adjusted using the software Webmax extended. Two dimensional images of calcein fluorescence were obtained under excitation at 488 nm. Tetramethylrhodamine ethylester (TMRE) and Rhod 2 fluorescence was imaged under excitation at 514 nm by confocal and epifluorescence microscopy as in our previous studies[8,13,14]. The time course of the whole cell fluorescence intensity was calculated with Aquacosmos software (Hamamatsu Photonics).

3. Results

Calcein fluorescence of H9c2 cells was images with confocal microscopy. Calcein fluorescence in intact H9c2 cells was uniform throughout the cells including the nucleus (Fig. 1A). Permeabilization of the cell membrane resulted in a decrease in the total fluorescence intensity; the residual fluorescence was present throughout the non-nuclear region with an elongated granular appearance (Fig. 1B). Both the appearance and intracellular localization of calcein fluorescence highly correlated with that of the mitochondria marker TMRE (Fig. 1C, 1D).

Effect of various interventions on the mitochondrial calcein fluorescence was examined with epifluorescence microscopy. Elevation of extramitochondrial Ca²⁺ concentration of the permeabilized H9c2 cells from 0 nM to 300 nM resulted in an increase in the fluorescence of the

Figure 1. Localization of calcein fluorescence to the mitochondria in digitonin-permeabilized H9c2 cells imaged by confocal microscopy. A: Calcein fluorescence before membrane permeabilization. B: Calcein fluorescence after permeabilization. C: TMRE fluorescence of the cells shown in B. D: Merge of calcein and TMRE fluorescence shown in B and C.

Figure 2. Effect of extramitochondrial Ca²⁺ elevation on mitochondrial Ca²⁺. Mitochondrial Ca²⁺ was monitored with Rhod-2 fluorescence and the extracellular Ca²⁺ was elevated during the period between the horizontal red lines.

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4. Discussion

In this study, we established a fluorescence imaging-based method for the evaluation of mPTP opening in cardiomyocyte-derived H9c2 cells using calcein fluorescence. Among the available fluorescent molecules, calcein has been used as the probe of choice to detect mPTP opening with imaging techniques [15]. Calcein has a suitable molecular mass (622 Da) and, despite its aromatic core, is highly hydrophilic because of its six negative and two positive charges at pH > 7 [16]. Although it is cell impermeant, calcein can be easily loaded into cells and organelles by using its acetomethoxy ester form. Finally, calcein does not undergo Ca²⁺-dependent changes in fluorescence at physiological pH, although its emission can be quenched by several metals, including Co²⁺ [16,17]. In the present study, cytosolic calcein fluorescence of permeabilized H9c2 cells was in good correlation with TMRE fluorescence (Fig. 1) indicating that the calcein fluorescence is localized to the mitochondria. The increase in cytoplasmic Ca²⁺ concentration, which causes an increase in mitochondrial Ca²⁺ concentration (Fig. 2), resulted in a decrease in mitochondrial calcein fluorescence (Fig. 3). This indicates that calcein escapes from the mitochondria through some transporting mechanism dependent on intramitochondrial Ca²⁺.

The mPTP forms from the F-ATP synthase, which can switch from an energy conserving device to an energy dissipating device through opening of a high conductance channel [10-12]. The mPTP allows unrestricted movement of solutes up to 1.5 kDa in size both in and out of the mitochondrial inner membrane. The opening of mPTP is triggered by various...
endogenous factors including mitochondrial Ca\textsuperscript{2+}. Cyclosporin A is a lipophilic cyclic peptide of 11 amino acids with a molecular weight of 1202 kDa, which is widely used as an immunosuppressant and anti-rejection drug in solid organ transplantation. When applied to cells, cyclosporin A inhibits mPTP opening through binding to cyclophilin D, a peptidyl-prolyl cis-trans isomerase which plays an important role in the mPTP opening\textsuperscript{[11,18]}. In the present study, the Ca\textsuperscript{2+}-induced decrease in mitochondrial calcein fluorescence was attenuated by cyclosporin A (Fig. 3), which indicates that the mPTP serves as the pathway for the escape of calcein from the mitochondria.

Mitochondria have specific transport pathways for Ca\textsuperscript{2+} uptake and release, such as the Ca\textsuperscript{2+} uniporter and the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger\textsuperscript{[19]}. Ca\textsuperscript{2+} influx into mitochondria occurs via the Ca\textsuperscript{2+} uniporter, which is driven by the negative charge of the mitochondrial membrane potential and inhibited by ruthenium red\textsuperscript{[19,20]}. Extrusion of mitochondrial Ca\textsuperscript{2+} is mediated primarily via mitochondrial Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger, which is activated by Na\textsuperscript{+} gradients between the matrix and the cytosol\textsuperscript{[19]} and is inhibited by diltiazem\textsuperscript{[21]}. We have shown in our pervious study that inhibition of the mitochondrial uniporter inhibited, while mitochondrial Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger inhibition enhanced, the rise in mitochondrial Ca\textsuperscript{2+} concentration on elevation of extramitochondrial Ca\textsuperscript{2+} concentration\textsuperscript{[14]}. In the present study, the extramitochondrial Ca\textsuperscript{2+}-induced decrease in mitochondrial calcein fluorescence was inhibited by ruthenium red and enhanced by diltiazem (Fig. 4). These results provide further pharmacological evidence that the mPTP opening was triggered by elevation of mitochondrial Ca\textsuperscript{2+}.

Cytoplasmic Ca\textsuperscript{2+} overload leads to cellular dysfunction through multiple pathways. There is increasing evidence suggesting that Ca\textsuperscript{2+} accumulation in the mitochondria is one of the major triggers for irreversible cell injury under pathological conditions such as myocardial ischemia\textsuperscript{[9]}. The results of the present study suggested that inhibition of Ca\textsuperscript{2+} influx to mitochondria during ischemia results in an inhibition of mPTP opening and may lead to preservation of mitochondrial function and cardioprotection. This hypothesis is supported by studies showing that agents such as ruthenium red or cyclosporine A, which either directly or indirectly prevent mPTP opening, enhances the recovery of following ischemia reperfusion\textsuperscript{[22,23]}. In contrast, inhibition of the mitochondrial Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger, which induced an increase in mitochondrial Ca\textsuperscript{2+} and mPTP opening during ischemia, was reported to produce deleterious effects during ischemia reperfusion including reduced resynthesis of energy phosphate\textsuperscript{[24]}.

In conclusion, the presently described fluorescence microscopy-based analyzing method provides a means to study the factors involved in the regulation of mitochondrial function in intact cells under physiological and various pathological conditions. Its contribution to the studies of lifestyle-related diseases is anticipated.

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6. Conflict of Interest
The authors declare that they have no conflict of interest concerning this study.

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心筋由来H9c2細胞におけるミトコンドリア Permeability Transition Pore 開口の蛍光イメージングによる観察系の構築

抄録
ミトコンドリア機能はエネルギー代謝の制御に中心的役割を果たしており、生活習慣病の発生・予防とも深く関連している。我々は蛍光イメージング法と透過性を高めた心筋由来H9c2細胞株を用いてミトコンドリアのpermeability transition poreの開口を観測する方法を確立した。細胞にcalceinを負荷し、digitonin処置により細胞膜の透過性を高めたH9c2細胞において、calceinの蛍光はミトコンドリアマーカーであるtetramethylrhodamine ethylesterの蛍光と局在が一致した。ミトコンドリア外液のCa²⁺濃度を上昇は、ミトコンドリア内Ca²⁺濃度を上昇させ、calcein蛍光を減少させた。この減少はpermeability transition pore阻害薬cyclosporin Aにより抑制された。また、ミトコンドリアuniporter阻害薬のruthenium redにより減弱し、ミトコンドリアNa⁺-Ca²⁺交換機構阻害作用を有するdiltiazemにより増強された。本研究で確立した蛍光イメージングによる解析法は、生理的条件下および各種生活習慣病の病態下でのミトコンドリア機能制御の研究に有用である。

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東邦大学大学院薬学研究科博士後期課程修了。
専門は循環薬理学。細胞内事象の蛍光イメージング法と薬理学的手法を組み合わせた総合的検討により、心臓機能を包括的に理解することを目指して研究を行っている。

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