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Voltage-dependent anion channel involved in the mitochondrial calcium cycle of cell lines carrying the mitochondrial DNA A4263G mutation

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ABSTRACT

In this study, we investigated the effects of the voltage-dependent anion channel (VDAC) on the mitochondrial calcium cycle in cell lines carrying the mitochondrial DNA A4263G mutation. We established lymphoblastoid cell lines from three symptomatic individuals and one asymptomatic individual from the large Chinese Han family carrying the A4263G mutation; these were compared with three control cell lines. The mitochondrial Ca^{2+} concentration and membrane potential were detected by loading cells with Rhod-2 and JC-1, respectively. Confocal imagines showed the average Rhod-2 and JC-1 fluorescence levels of individuals carrying the $tRNA^{Ile}$ A4263G mutation were lower than those of the control group ($P < 0.05$). The baseline Rhod-2 fluorescence in the control group increased after exposure to atractyloside (an opener of the adenine nucleotide translocator, $P < 0.05$), but no significant change was detected in the cell line harboring the A4263G mutation ($P > 0.05$). The baseline JC-1 fluorescence in both the mutated and control cell lines decreased after subsequent exposure to atractyloside ($P < 0.05$), whereas this effect of atractyloside was inhibited by Cyclosporin A (CsA, a VDAC blocker). We conclude that the mitochondrial VDAC is involved in both the increase of mitochondrial permeability to Ca^{2+} and the decrease of mitochondrial membrane potential in cell lines carrying the mtDNA A4263G mutation.

1. Introduction

Cardiovascular disease is the leading cause of death in America and throughout the world. Cardiovascular disease includes high blood pressure, coronary heart disease, heart failure, and stroke. In particular, hypertension affects approximately one billion individuals worldwide and 130 million individuals in China [1]. We previously reported that some families showed an obvious pattern of maternal inheritance that was indicative of a mitochondrial disorder [2–5]. In a previous study on a large Chinese Han family with maternally-inherited hypertension, we identified a mutation in the mitochondrial $tRNA^{Ile}$ A4263G gene (Fig. 4). However, how the mechanism of these mutations contributes to hypertension is still unclear.

Mitochondria affect cytoplasmic Ca^{2+} metabolism in two ways: indirectly via mitochondrially produced ATP that is used by Ca^{2+}-dependent ATPase proteins to pump Ca^{2+} out of the cell or into the intracellular stores, such as the sarcoplasmic and endoplasmic reticula, and directly via the mitochondrial membrane potential which drives the uptake of Ca^{2+} into mitochondria through a Ca^{2+}-uniporter. The uptake of Ca^{2+} by the mitochondria acts both as a buffer, modulates intracellular calcium signals and a sink for excess cytoplasmic Ca^{2+} and prevent calcium overload. Numerous observations indicate that collapse of the mitochondrial membrane potential triggers cell death, whereas stabilization of the mitochondrial membrane potential prevents cell death.

The currently available data support the hypothesis that the permeability transition (PT) accounts for the death-associated mitochondrial membrane dissipation [6]. PT is now thought to be regulated by multi-protein ensembles, including cytosolic proteins (hexokinase), peripheral benzodiazepine receptors (PBR), voltage-dependent anion channels (VDAC), creatine kinases, adenine nucleotide translocators (ANT) and matrix proteins (cyclophilin D), at inner/outer membrane contact sites [7,8]. To study the effects of VDAC on the mitochondrial calcium cycle of the cell lines carrying the mtDNA 4263A→G mutation, we established lymphoblastoid cell lines [9] derived from three symptomatic and one asymptomatic hypertensive individuals in the family carrying the A4263G mutation, and we compared them to three control cell lines. Confocal imaging was used to evaluate the mitochondrial calcium changes of the cell lines carrying the $tRNA^{Ile}$ A4263G

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mutation after exposure to CsA [10] (a blocker of VDAC) and atractyloside (an opener of ANT). The mitochondrial VDAC was involved in the change of both the mitochondrial membrane potential and the mitochondrial permeability to Ca^{2+} in the cell lines carrying the mtDNA A4263G mutation.

2. Materials and methods

2.1. Cell lines and culture conditions

Blood samples were obtained from all of the participating family members. The participants gave informed consent, and blood was collected according to the protocols approved by the ethics committee of the Chinese PLA General Hospital and the Cincinnati Children's Hospital Medical Center Institute Review Board. Lymphoblastoid cell lines were immortalized by transformation with the Epstein-Barr virus, as described previously [9]. The cell lines derived from the three members of the Chinese Han family (three individuals [II-4, III-14, and III-18] with hypertension and one asymptomatic individual [III-19]) and three genetically unrelated control individuals (A1, A2, and A3) were grown in RPMI 1640 medium (Gibco) that was supplemented with 15% fetal bovine serum (FBS).

2.2. $[Ca^{2+}]_m$ and mitochondrial membrane measurements

The mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_m$) was measured by loading cells with the Ca^{2+} fluorophore Rhod-2. Lymphoblastoid cells were loaded with 10 μmol/L rhod-2 acetoxymethyl ester for 120 min at 4 °C and then incubated for 30 min at 37 °C in the culture medium. This 2-step cold loading/warm incubation protocol achieves exclusive loading of rhod-2 into the mitochondria [11]. The mitochondrial membrane potential was monitored with the fluorescent probe JC-1. Lymphoblastoid cells were incubated with 0.5 μmol/L JC-1 for 10 min at 37 °C [12]. Cells loaded with rhod-2 or JC-1 were perfused with normal Tyrode's solution (37 °C) containing the following components: 140 mmol/L NaCl, 5.4 mmol/L KCl, 2.7 mmol/L $CaCl_2$, 0.33 mmol/L NaH_2PO4, 0.5 mmol/L MgCl2, 5 mmol/L HEPES, and 5.5 mmol/L glucose pH 7.4. Rhod-2 fluorescence was excited at 540 nm, and the emission was monitored through a 605 nm (55 nm bandpass) barrier filter. JC-1 was excited at 488 nm, and the red emission fluorescence was detected with the use of a 580 nm long-pass filter.

2.3. Statistical analysis

Data are expressed as $\overline{X} \pm SD$. Statistical comparisons were made using Student's t test. A value of $P < 0.05$ was considered to be significant. The statistical analysis was performed by SPSS (v.11.0, SPSS Inc., Chicago, IL).

3. Results

3.1. $[Ca^{2+}]_m$ by confocal imaging

Several reports indicated that channel blockers and openers, such as 5-hydroxydecanoate and diazoxide, affected the mitochondrial calcium cycle. In the current study, we found that Rhod-2 fluorescence (which indicates $[Ca^{2+}]_m$) of lymphoblastoid cells in the control group and suspended in Tyrode's solution increased after exposure to 5 μM atractyloside (119.5 + 19% of the baseline, $P < 0.05$) and was inhibited by 2 μM CsA (see Figs. 1 and 3). This effect of atractyloside was not observed in the lymphoblastoid cells carrying the $tRNA^{Ile}$ A4263G mutation. Conversely, the $[Ca^{2+}]_m$ of lymphoblastoid cells carrying the $tRNA^{Ile}$ A4263G mutation increased after exposure to CsA.

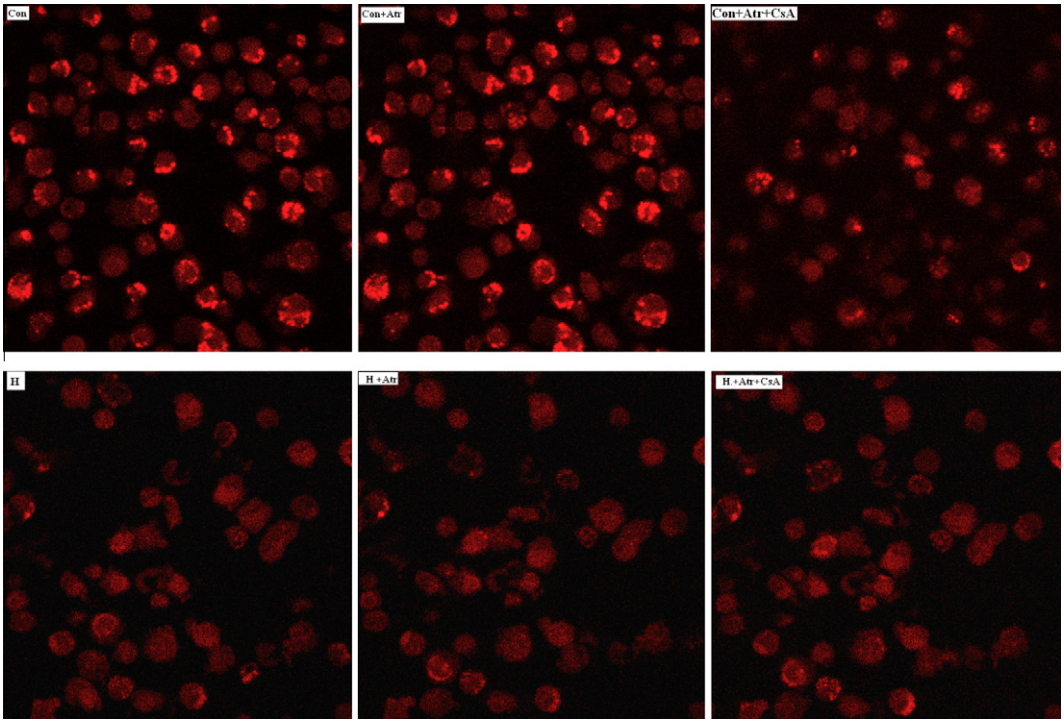

Fig. 1. Two-dimensional confocal images of Rhod-2 fluorescence. Atr (atractyloside, 5 μM), CsA (Novartis AG, 2 μM), Con: the control cell, Con + Atr: the control cells treated with atractyloside, Con + Atr + CsA: the control cells treated with Atr and CsA, H1: III-14 cell line, H1 + Atr: III-14 cell line treated with Atr, H1 + Atr + CsA: III-14 cell line treated with Atr and CsA.

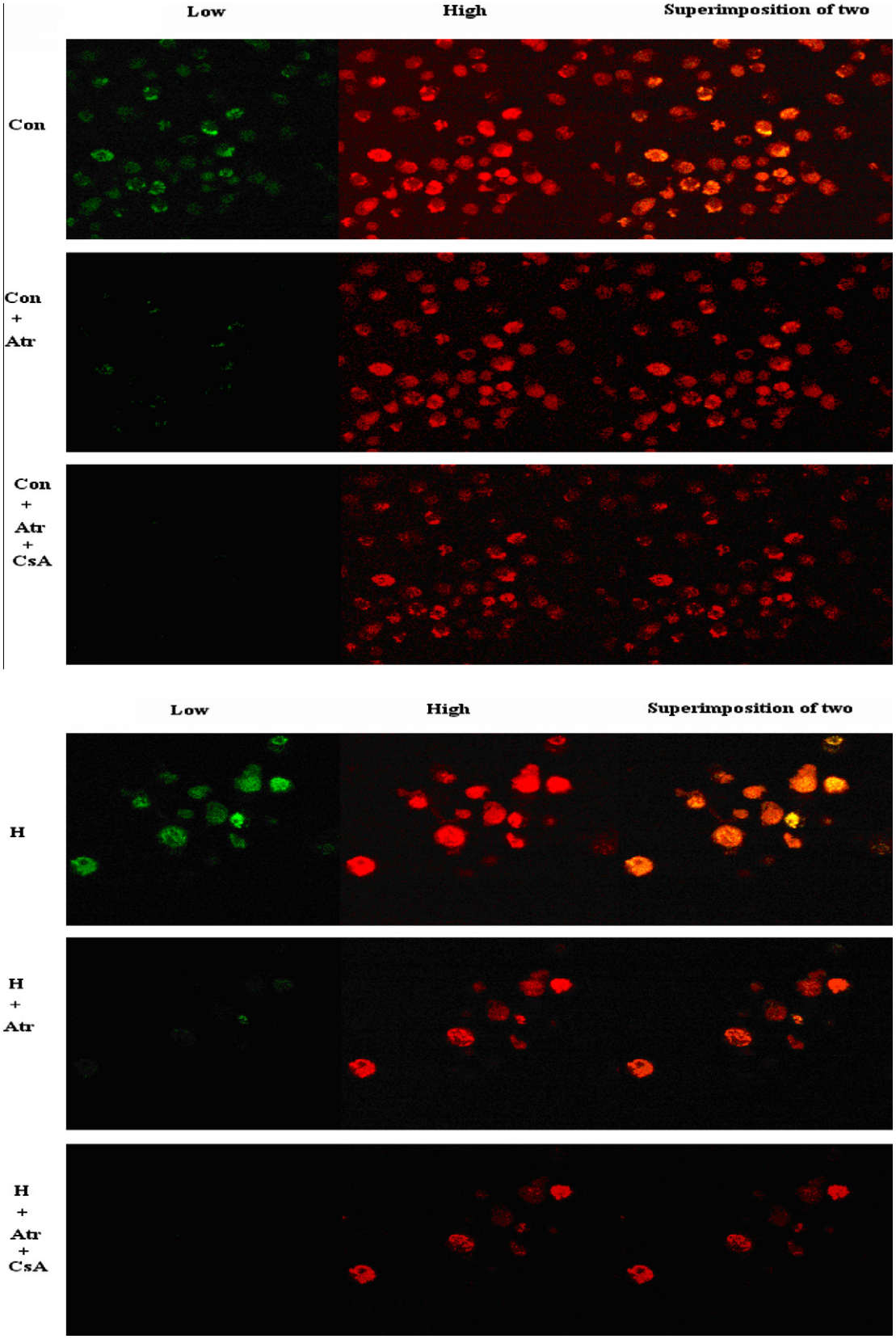

Fig. 2. Two-dimensional confocal images of JC-1 fluorescence. Atr: atractyloside (5 μM), CsA (2 μM). Con: the control group, Con + Atr: the control cells treated with Atr, Con + Atr + CsA: the control cells treated with Atr and CsA, H1: III-14 cell line, H1 + Atr: III-14 cell line treated with Atr, H1 + Atr + CsA: III-14 cell line treated with Atr and CsA.

3.2. $\Delta\Psi_m$ measured by confocal imaging

The membrane potential ($\Delta\Psi_m$) of the cell lines was detected by loading JC-1 into the mitochondria. Two-dimensional confocal imaging of Rhod-2 fluorescence showed that the $\Delta\Psi_m$ of the cell line carrying the mtDNA $tRNA^{Ile}$ A4263G mutation decreased (123.4% of the baseline, $P < 0.05$) after exposure to 5 μM atractyloside and was inhibited by CsA (2 μM). This change was consistent with the control cell lines (see Figs. 2 and 3).

4. Discussion

The physiological function of the mitochondrion depends partly on the mitochondrial membrane potential and includes the electronic transmission and synthesis of ATP. When compared with the cell membrane potential, the mitochondrial membrane potential is ranged from −150 to −180 mV and the pump of the synthesis of ATP. Ca^{2+} directly through the mitochondrial membrane potential drives the uptake of Ca^{2+} into the mitochondria through a Ca^{2+}-uniporter. The Ca^{2+} transporter is a complex channel, and its specific molecular structure, which has been previously inhibited, allows it to open or close based on a membrane threshold effect. [13] The accumulation of Ca^{2+} in the mitochondria would lead to mitochondrial swelling, eventually inducing apoptosis. Therefore, to maintain the homeostasis of Ca^{2+}, it must be continuously pumped out of the mitochondria through Na^+/Ca^{2+} transporters on the mitochondrial membrane. In all cell types, calcium signals are transmitted into the mitochondria as a result of processes, such as the TCA (tricarboxylic acid) cycle and ATP synthesis.

Previous research has shown that Ca^{2+} exchange is similar in the mitochondria, the endoplasmic reticulum (ER), the sarcoplasmic reticulum (SR) and the plasma membrane. The uptake of Ca^{2+} by mitochondria serves as a buffer, modulates intracellular calcium signals, and may also act as a sink for excess cytoplasmic Ca^{2+}, thereby protecting the cell from calcium overload. Because many researchers have shown that mitochondrial calcium overload is involved in a variety of diseases, studies on the mitochondrial "calcium cycle" have become popular. Current data indicate that the channels involved in the "calcium cycle" include mitochondrial calcium-activated potassium channels ($mitoK_{Ca}$), mitochondrial ATP-sensitive potassium channels ($mitoK_{ATP}$) and mitochondrial permeability transporters (mPTP). Each channel has specific stimulators and inhibitors. [14].

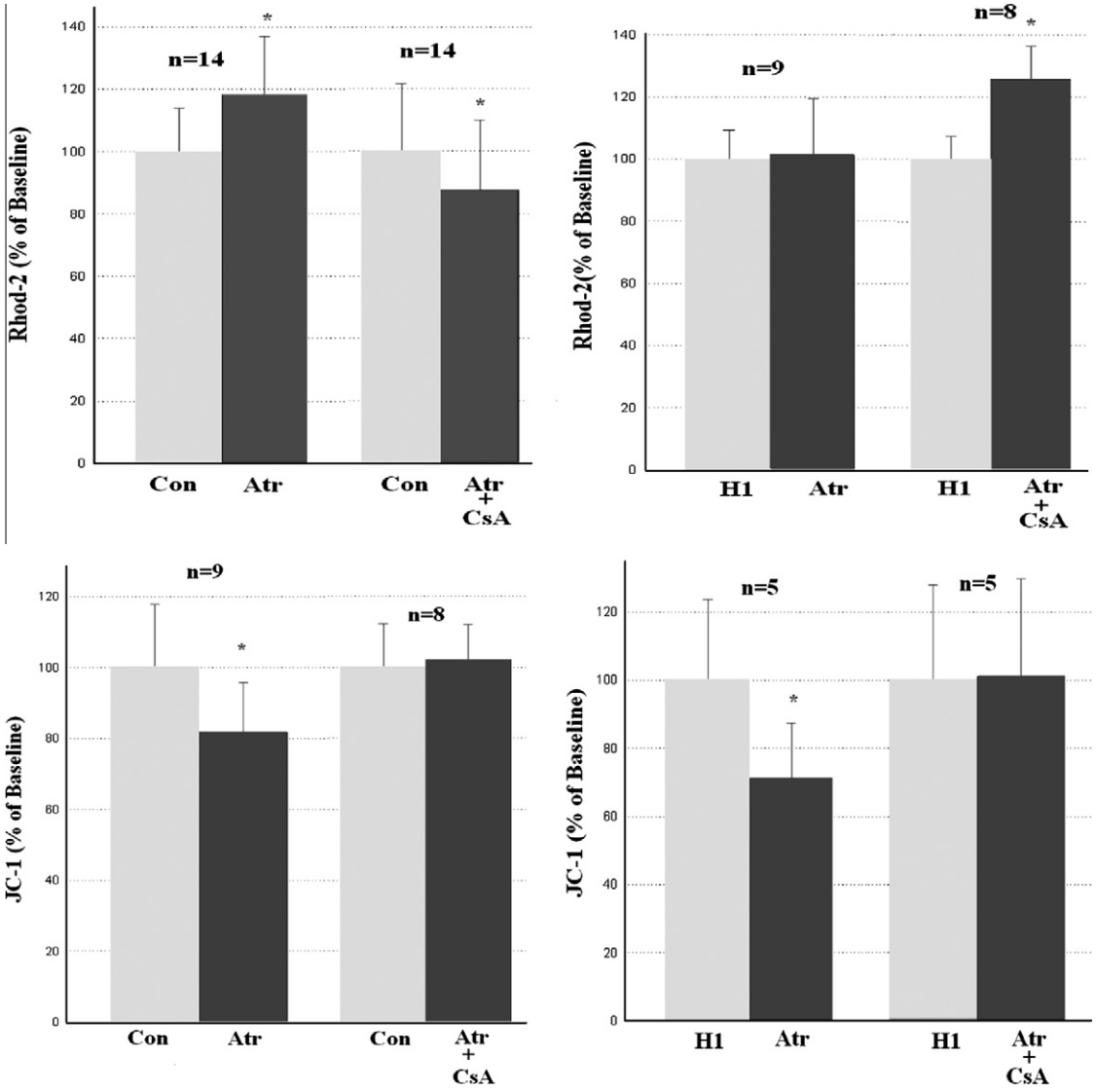

Fig. 3. Summary of the relative changes of rhod-2 and JC-1 fluorescence. Con: control, H1: III-14, Atra: atractyloside, 5 μM, CsA 2 μM. *$P < 0.05$ compared with control had significant difference.

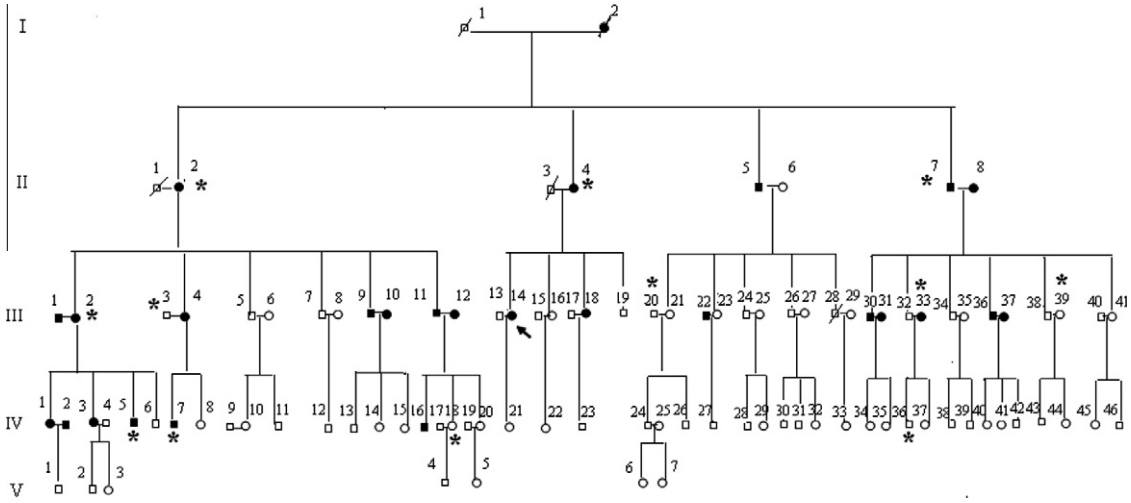

Fig. 4. The Han Chinese pedigree with a maternal pattern of inheritance of hypertension. Affected individuals are indicated by filled symbols. The arrowhead denotes the proband. ∗ denotes cardiac hypertrophy.

The mPTP was involved in the mitochondrial damage induced by Ca^{2+} overload [15]. It is composed of six components, the most important of which are the ANT, located in the inner mitochondrial membrane, and the VDAC, located in the outer mitochondrial membrane. In cases of mitochondrial Ca^{2+} overload, studies [16,17] have shown that the mPTP was opened, and Ca^{2+} was released into the cytoplasm. They also have shown that the excessive opening of the channel led to mitochondrial swelling, mitochondrial membrane collapse and the release of compounds, such as Cyt C and AIF, into the cytoplasm; these processes initiate programmed cell death. In addition, the mitochondrial membrane is necessary for ATP synthesis [15]. If the channel is irreversibly opened, it leads to cell death. Previous studies have found that CsA can inhibit the channel opening, reduce the area of ischemia and promote the uptake of deoxyglucose into the mitochondria [18]. mPTP has been a target drug for myocardial protection.

This study showed that the $[Ca^{2+}]_m$ and the mitochondrial membrane potential of mutated cell lines was significantly lower than those of the control group. After exposure to atractyloside, the $[Ca^{2+}]_m$ of the control cell line increased and the mitochondrial membrane potential decreased significantly. It is understandable that atractyloside, an opener of ANT, could increase the permeability of the PT, which led to both a decrease in the mitochondrial membrane potential and an increase in the $[Ca^{2+}]_m$. In cell lines carrying the $tRNA^{Ile}$ A4263G mutation, the mitochondrial membrane potential increased after exposure to atractyloside, whereas the $[Ca^{2+}]_m$ did not change significantly. Conversely, exposure to CsA caused an increase in the $[Ca^{2+}]_m$. In non-excitable cells, the change in the mitochondrial membrane potential after exposure to atractyloside (an ANT opener) drives the uptake of Ca^{2+} into the mitochondria and causes mitochondrial membrane depolarization. This change could be inhibited partly by CsA, which is a VDAC inhibitor. However, this change was not observed in the lymphocyte cell lines from the hypertensive patients carrying the mtDNA mutation. We speculate that ANT had been completely opened, causing the collapse of the mitochondrial membrane potential. As a result, the channel could not further open after exposure to atractyloside. However, this does not explain why the mitochondrial membrane potential decreased. We speculate that other channels may be involved in changes in mitochondrial membrane potential. Finally, we conclude that the mitochondrial VDAC is involved in the increase in the mitochondrial permeability to Ca^{2+} and the decrease in the mitochondrial membrane potential of the cell lines carrying the mtDNA A4263G mutations.

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