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

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Research on voltage-dependent anion channel (VDAC) has accelerated as evidence grows of its importance in mitochondrial function and in apoptosis. In this report, we studied the effect of VDAC contributed to the apoptosis of the cell lines carrying mitochondrial DNA A4263G mutation. We established lymphoblastoid cell lines derived from 3 symptomatic and 1 asymptomatic hypertension individuals in the family carrying A4263G mutation compared with 3 control cell lines. The mitochondrial potential ($\Delta\Psi_m$) and apoptosis was detected by flow cytometry and the co-localization of VDAC and Bax was evaluated by confocal microscopy. The results showed that the expression of VDAC and Bax of the lymphoblastoid cell lines carrying mtDNA A4263G mutation increased compared with control group, while the expression of small conductance calcium dependant potassium (sK$_{Ca}$) had no change. The confocal imaging showed co-localization of VDAC/Bax on the outer membrane of mitochondrial of the cell lines from individuals carrying mtDNA A4263G mutation, while the interaction was not seen on control group. Flow cytometry showed mitochondrial potential of cell lines from individuals carrying mtDNA A4263G mutation decreased 32%, while the apoptosis increased compared with control group ($P<0.05$). This difference was attenuated by Cyclosporin A (CsA, 2$\mu$M), a blocker of VDAC. In conclusion, the change of expression of mitochondrial VDAC and subcellular co-localization of VDAC/Bax leads to the significant increase of mitochondrial permeability and apoptosis in the cell lines carrying mtDNA A4263G mutations.

**Key words**: mitochondrial DNA (mtDNA); VDAC; apoptosis; mitochondrial potential; mutation
**Introduction**

Hypertension is one of the most common cardiovascular diseases associated with coronary heart disease, stroke, congestive heart failure and renal dysfunction, and the major modifiable risk factor of poor prognosis of a variety of cardiovascular diseases. Essential hypertension is influenced by multiple environmental and genetic factors with the latter contributing to 30–60% etiology \[^1\]. During clinical works we found essential hypertension presented familial congregation, some of them had obvious pattern of maternal inheritance, which was a hallmark of mitochondrial disorders \[^2-5\]. Our previous study found a large Chinese Han family with typical maternal inheritance hypertension. Biochemical study revealed A4263G mutation in the tRNA\(^{\text{Ile}}\) gene, which was extraordinarily conserved base in every sequenced isoleucine tRNA from bacteria to human mitochondrial and could influence the combination of amino acids with tRNA \[^6\].

Recently, there were several studies reported voltage-dependent anion channel (VDAC) located on the out-member of mitochondrial was associated with type 2 diabetes mellitus \[^7-9\], which was very important on the pathogenesis of mitochondrial diseases \[^10, 11\]. The voltage-dependent anion channel (VDAC) controls the passage of adenine nucleotides, Ca\(^{2+}\), and other metabolites \[^12, 13\] into and from mitochondria. Recent studies have shown that VDAC also plays a role in apoptosis as a constituent of the mitochondrial permeability transition pore (PTP) \[^14-16\]. To study on the mechanism of tRNA\(^{\text{Ile}}\) A4263G mutation on high blood pressure, we established lymphoblastoid cell lines from 3 symptomatic and 1 asymptomatic individuals in this family carrying A4263G mutation and 3 control individuals. Since VDAC has effect on many mitochondrial diseases that we supposed that VDAC might be involved in apoptosis of cell lines carrying tRNA\(^{\text{Ile}}\) A4263G mutation.

**Materials and methods**

1.1 Cell lines and culture conditions

Lymphoblastoid cell lines were immortalized by transformation with the Epstein-Barr virus, as described elsewhere \[^17\]. Cell lines derived from four members of the Chinese family (three individuals [II-4, III-14, III-18] with hypertension, one
individual [III-19] without symptom, see fig. 1), and three genetically unrelated control individuals (A1, A2, A3) were grown in RPMI 1640 medium (Gibco), supplemented with 15% fetal bovine serum (FBS).

1.2 Expression of VDAC, Bax and sK\(_{Ca}\)

Cells lines carrying mtDNA tRNA\(^{\text{Ile}}\) 4263 A→G and control groups were washed with ice-cold PBS once and total RNA was isolated with TRIzol (Invitrogen) according to the instructions of the manufacturer. About 2 µg RNA were treated with ribonuclease-free deoxyribonuclease, and cDNA was synthesized by using Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA), and 2 µl of synthesized cDNA was subjected to 44 cycles of PCR that resulted in a single specific amplification product of the expected size. The PCR conditions were as follows: 30 sec denaturation at 94 °C, 1 min annealing at 55°C (VDAC, Bax) or 58 °C (sK\(_{Ca}\)), and 45 sec extension at 72°C. PCR primers used in this study are as follows: VDAC sense 5’-CTGAGTACGGCCTGACGTTT-3’ and antisense 5’-ACTCTGTCCCGTCATTCACA-3’; Bax, sense 5’-GCAGCTTAACGCACCAAT TA-3’ and antisense 5’-CAGTTGAAGTTGCCGTCAGA-3’; sK\(_{Ca}\), sense 5’-GCAGCTTAACGCACCAATTA-3’ and antisense 5’-TGAGGGAAAGGACCAC TGAT-3’. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control of the PCR. The primers for GAPDH were sense 5’-CTGCACCACCAACTGCTTAG-3’ and antisense 5’-TTCAGCTCAGGGATGACC TT-3’.

To determine the specificity of RT-PCRs, we performed the amplifications three times and took the mean value of these three reactions. The RT-PCRs were in the linear range of amplification for the target mRNA as well as for the control. Each RT-PCR product was demonstrated on 1.5% agarose gel stained with ethidium bromide. The bands were normalized with internal control GAPDH. To evaluate the function of CsA (an inhibitor of VDAC) on the expression of VDAC, Bax and sK\(_{Ca}\), cell lines were incubated with CsA (Novartis, 2µM) for 16 hours and then tested.

1.3 Confocal microscopy

A fluorescent double staining was performed to visualize the co-localization between
Bax and VDAC-1. Cells grown on glass cover slips in 6-well plates were fixed with 4% paraformaldehyde in PBS. After blocked with 5% BSA for 15 min, the cover slips were incubated with primary antibody (VDAC, 1:100, 200µl Polyclonal Antibody, Cell signaling; Bax, Polyclonal Antibody, NeoMarkers) overnight, followed by fluorescent labelled secondary antibody for 60 min at 37°C. After several washes, mounted onto the glass slides, all the cover slips were imaged using confocal laser scanning system (Bio-Rad Co., RADIANCE 2100, USA). The optimal excitation-emission wavelength combination: Argon 488 nm laser with 505-525 nm filter for Alexa Fluor 488, and HeNe 543 laser with 610 nm filter for Alexa Fluor 546.

1.4 Mitochondrial membrane potential and apoptosis measurements

The mitochondrial membrane potential ($\Delta \Psi_m$) was monitored with the fluorescent probe JC-1. Lymphoblastoid cell lines were incubated with 0.1µM JC-1 (ALEXIS biochemical) for 10 minutes at 37°C. After the loading period the cells were rinsed with phosphate-buffered saline/bovine serum albumin and resuspended in 0.1µM JC-1 in phosphate-buffered saline/bovine serum albumin at room temperature. The $\Delta \Psi_m$ of cells was measured by cytofluorimetry (FL3). To evaluate the function of CsA on the mitochondrial membrane potential, cell lines were incubated with CsA (Novartis, 2µM) for 30mins and then tested the change of the mitochondrial membrane potential. Every sample was rinsed five times with phosphate-buffered saline (PBS). To detect the apoptosis of autophagic cell death with Annexin V/FITC and PI stain by using laser scanning confocal microscopy (LSCM).

1.5 Statistical analyses

All data were presented as $\bar{X} \pm$ SD. Comparison of continuous variables was performed with unpaired Student’s $t$ test. Statistical significance was accepted when $P$ was <0.05. Statistic analysis was performed by SPSS (version 11.0).

2 Results

2.1 Expression of VDAC, Bax and sK$_{Ca}$

We compared the expression of VDAC, Bax and sK$_{Ca}$ of the cell lines carrying
mtDNA tRNA\textsuperscript{Ile} 4263 A→G mutation with the control group, and evaluated the effect of CsA. The results showed that the expression of VDAC and Bax of the cell lines carrying mtDNA tRNA\textsuperscript{Ile} 4263 A→G mutation increased 120.2% and 117.3% respectively compared with control cell lines, while sK\textsubscript{Ca} had no change. After incubated with CsA for 16 hours, the expression of VDAC decreased 69.3%, while Bax and sK\textsubscript{Ca} had no change (fig. 2).

2.2 Co-localization of VDAC and Bax protein

Co-localization of apoptosis-regulating proteins at the subcellular level was visualized on the confocal images in representative cell cross-sections in Bax/VDAC (see fig. 3). We know that VDAC and Bax will show green and red fluorescence respectively if they had no interaction. In comparison with control cells where no pattern of interaction was observed, the cell lines (III-14, III-19) carrying mtDNA tRNA\textsuperscript{Ile} A4263G mutation presented the pattern of co-localization between Bax and VDAC revealed as a strong yellow fluorescence emitted as a result of simultaneous excitation of both green and red fluorescence dyes. After incubated with CsA for 30 min, the confocal images of the cell lines carrying mtDNA tRNA\textsuperscript{Ile} A4263G mutation presented green and red fluorescence separately which suggested VDAC and Bax protein had no interaction after incubated with CsA.

2.3 Mitochondrial membrane potential (\(\Delta\Psi_m\)) and apoptosis

Several studies reported that the \(\Delta\Psi_m\) was influenced by mitoK\textsubscript{ATP}, mitoK\textsubscript{Ca} and mitochondrial permeability transition pore (PTP) \[20\]. Cytometry images of lymphocytes loaded with JC-1, showed the \(\Delta\Psi_m\) of cell lines carrying mtDNA tRNA\textsuperscript{Ile} A4263G mutation decreased 32% compared with control group \((P<0.05)\). After incubated with CsA for 30 min the \(\Delta\Psi_m\) of both control and the mutated cell lines increased (the control group 33.6%, III-14 84.4%, III-19 137.7%, see fig. 4). After incubated with CsA the \(\Delta\Psi_m\) was improved, especially for the cell line carrying mtDNA tRNA\textsuperscript{Ile} A4263G mutation (see fig. 5). The apoptosis of cell lines carrying tRNA\textsuperscript{Ile} A4263G mutation gene increased 30%. The apoptosis of both the control group and the patients were decreased by CsA, the control group decreased 24.6%, III-14 56.9% and III-19 67.1%. (see fig. 6)
3 Discussions

Hypertension is one of the main risk factors associated with cardiovascular death, affecting approximately 1 billion individuals worldwide and 130 million in China and the morbidity of essential hypertension (EHT) is still increasing \[^{21}\]. Epidemiological studies have demonstrated that estimates of genetic variance range from 30-60% \[^{1}\]. Molecular genetic studies have identified mutations in nuclear genes and mitochondrial DNA (mtDNA), which have characteristics of maternal inheritance \[^{22-25}\]. More recently Framingham heart study \[^{26}\] showed quantitative blood pressure were analyzed on 6421 participants from 1593 families and estimated heritability due to maternal effects was 5% for multivariable-adjusted long-term average systolic blood pressure. The heritability of diastolic blood pressure due to maternal effects was 4%.

Our previous studies found a large Chinese Han family with typical maternally inherited hypertension and the sequence analysis revealed A4263G mutation in the tRNA\[^{\text{Ile}}\] gene, which was extraordinarily conserved base in every sequenced isoleucine tRNA from bacteria to human mitochondrial. And the segregation analysis also proved this family was maternal inheritance. Voltage-dependent anion channel (VDAC) was a high conserved protein which located on the mitochondrial outer membrane \[^{11}\]. VDAC participates in mitochondrial metabolism by conveying ATP and ADP out/into mitochondrial membrane. ATP and ADP must pass though VDAC/ANT (adenine nucleotide translocator) \[^{11}\]. VDAC closure inhibits the release of ATP from the mitochondrial which promotes opening of sensitive K\(_{\text{ATP}}\) channels in the plasma membrane. VDAC as the outer mitochondrial membrane protein, providing the pathway for Ca\(^{2+}\) transport into and out of mitochondrial, must thereby have a role in intracellular Ca\(^{2+}\) signaling \[^{27}\]. VDAC is the key point on calcium homeostasis. \(\alpha\)Bid, a pro-apoptotic Bcl\(_2\) member, closes VDAC, which may account for VDAC inhibition during apoptosis, at least in part \[^{28}\]. In contrast, anti-apoptotic Bcl\(_2\)-XL prevents VDAC closure, which is consistent with the notion that VDAC opening is anti-apoptotic \[^{29}\]. VDAC also appears to be an anchoring point for pro- and anti-apoptotic proteins and has been proposed to be part of the cytochrome c
release channel in the outer membrane that forms in apoptosis. Our study found VDAC expression of cell lines carrying mtDNA tRNA\textsubscript{Ile} A4263G mutation decreased significantly while the exact mechanism was still unclear, which may be associated with dysfunction of energy metabolism and increase of ROS\textsuperscript{[30]}. Subcellular co-localization showed VDAC combined with Bax protein on the cell lines carrying mtDNA tRNA\textsubscript{Ile} A4263G mutation associated with the decrease of $\Delta \Psi_m$ and increase of apoptosis, which was not seen on the control group. Apoptosis was decreased after incubated with CsA by inhibit the combination of VDAC and Bax.

To date, efforts to identify hypertension gene have focused on the genetic markers and candidate genes in the nuclear genome. Although many researches have revealed the genetics of EHT, the pathophysiological mechanisms that underlie essential hypertension remain mysterious. Maternally inherited hypertension may be the result of interaction of mtDNA and nuclear DNA mutation. Much unknown field on potential role for mtDNA mutation in EHT needs to be investigated in future.
References:

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Fig. 1 The Han Chinese pedigree with hypertension. Affected individuals are indicated by filled symbols. Arrowhead denotes proband. * denotes cardiac hypertrophy.
Fig. 2 Expression of VDAC, Bax and sK$_{Ca}$ mRNA. Con: cell line from control group; H$_{1}$: cell line from III-14; CsA: cell line from III-14 after incubated with CsA (2µM) for 16 hours. A: the expression of VDAC mRNA; B: the expression of Bax mRNA; C: the expression of sK$_{Ca}$ channel mRNA.
Fig. 3 Localization of mitochondrial VDAC and Bax by confocal microscopy. Control: cell line from control group; Con+CsA: cell line incubated with CsA (2µM) for 30 mins; H-1: cell line from III-14; H-1+CsA: cell line incubated with CsA (2µM) for 30 mins; H-4: cell line from III-19; H-4+CsA: cell line incubated with CsA (2µM) for 30 mins. VDAC was stained with FITC-green (green fluorescence); Bax with rhodamine 123 (red fluorescence).
Fig. 4 Mitochondrial potential ($\Delta \Psi_m$) of cell lines from III-14, III-19 and control group by flow cytometry. Left and right figure showed $\Delta \Psi_m$ detected by flow cytometry before and after incubated with CsA (2µM) for 30 mins, respectively. A: cell lines of control group; B: III-14; C: III-19.
Fig. 5 Comparison of $\Delta \Psi_m$ of the cell lines between the patients and the control group. The average value of $\Delta \Psi_m$ of cell lines from patients with tRNA$^\text{Ile}$ A4263G mutation gene was decreased 32% compared with that of the control group; $\Delta \Psi_m$ of both the control group and the patients were improved by CsA, the control group increased 33.6%, III-14 84.4% and III-19 137.7%.
Fig 6. The apoptosis of cell lines from III-14, III-19 and control group by flow cytometry. Left and right figure showed the apoptosis detected by flow cytometry before and after incubated with CsA (2\textmu M) for 30 mins, respectively.
Additional files provided with this submission:

Additional file 1: to the editorial board.doc, 26K
http://www.biomedcentral.com/imedia/1613630072227816/supp1.doc