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Research Article

Mitochondrial permeability transition triggers the release of mtDNA fragments

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Abstract. Fragments of mitochondrial DNA are released from mitochondria upon opening of the mitochondrial permeability transition pore. Cyclosporin A, an inhibitor of pore opening, completely prevented the release of mitochondrial fragments. Induction of mitochondrial permeability transition and subsequent release of the fragments of mitochondrial DNA could be one cause of genomic instability in the cell.

Key words. mtDNA; mitochondria; calcium; cyclosporin A; membrane potential.

The nuclear genome routinely contains numerous scattered fragments of mitochondrial genes. In fact, mitochondrial DNA (mtDNA) sequences in the nuclear genome have been observed in all eukaryotes studied, but the number of these mitochondrial fragments varies among different species [1, 2]. Most of the mtDNA sequences integrated into the nuclear genome were recently identified [3].

Mitochondria are both a major source of oxidants and a target for their damaging effects. Even under normal physiological conditions, the mitochondrial electron transport chain is a primary source of reactive oxygen species (ROS) within the cell due to the leakage of unpaired electrons, which in turn partially reduce molecular oxygen, as they are being transported down the respiratory complexes [4, 5]. Mitochondrial-generated ROS trigger the formation of oxidative products such as 8-hydroxydeoxyguanosine, a lesion arising as a result of oxidative DNA damage. mtDNA is especially susceptible to attack by these ROS because it lacks protective histones and is in close proximity to the electron transport chain, a significant locus for free-radical production [4, 5]. However, the mechanism by which fragments of mtDNA are released from mitochondria is still unknown.

Here, we show that mtDNA can be released from mitochondria upon opening of a non-specific pore in the inner mitochondrial membrane. This phenomenon was described by Haworth and Hunter at the end of the 1970s in a seminal series of papers [6–8] in which they showed that Ca^{2+} uptake stimulates drastic changes in mitochondrial morphology and functional activity due to the opening of a non-specific pore in the inner mitochondrial membrane, commonly known as the mitochondrial permeability transition (MPT) pore.

According to a current opinion, the MPT pore is formed by the association of several proteins located at contact sites between the inner and outer mitochondrial membranes. These are the outer membrane voltage-dependent anion channel, the inner-membrane adenine nucleotide translocase, and cyclophilin D [for a reviews see refs. 9, 10]. Mitochondrial Ca^{2+} accumulation is obligatory for MPT induction, although the sensitivity of MPT to Ca^{2+}

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can be significantly enhanced by various factors. Among these are the depletion of adenine nucleotides, an elevated level of inorganic phosphate and oxidative stress [9, 10].

In the present paper we show that the induction of the MPT by calcium in the presence of inorganic phosphate triggers the release of mtDNA fragments. Cyclosporin A (CsA), an inhibitor of pore opening, prevents both MPT induction and subsequent release of DNA fragments from mitochondria.

Materials and methods

Isolation of mouse liver mitochondria

The liver of a 6- to 8-week-old Balb/c mouse was minced on ice, resuspended in 10 ml ice-cold isolation buffer (250 mM sucrose, 1 mM EDTA, 10 mM Hepes-KOH, pH 7.4), and homogenized with a glass Dounce homogenizer and Teflon pestle. Homogenates were centrifuged at 600 g for 10 min at 4 °C to remove cellular fragments and blood. The supernatant fraction was then centrifuged at 6000 g for 10 min at 4 °C. After centrifugation, supernatants and floating lipid layers were aspirated, and the mitochondrial pellet was resuspended in isolation buffer without EDTA and centrifuged at 6000 g for 10 min at 4 °C. The final mitochondrial pellet was resuspended in isolation buffer at a protein concentration of 65–70 mg/ml. Fresh mitochondria were prepared for each experiment and used within 4 h.

Estimation of functional activity of isolated mitochondria

Induction of the MPT was assessed by a decrease in the mitochondrial membrane potential ($\Delta\psi$). Estimation of $\Delta\psi$ was performed using an electrode sensitive to the lipophilic cation tetraphenylphosphonium (TPP^+). Energized mitochondria rapidly accumulate TPP^+ from the incubation buffer and release this cation as $\Delta\psi$ decays. Mitochondria (1 mg/ml) were incubated in a buffer containing 150 mM KCl, 5 mM KH_2PO_4, 5 mM succinate, 2 µM TPP^+, and 10 mM HEPES, pH 7.4. Oxygen consumption by isolated mitochondria was measured using a Clark-type oxygen electrode (Yellow Spring Instrument Co.) at 22 °C. Mitochondria with a respiratory control ratio (defined as the rate of respiration in the presence of ADP divided by the rate obtained following the expenditure of ADP) above 4 were used for all experiments.

DNA extraction

For DNA extraction, mitochondria were first pelleted by centrifugation at 12,500 g for 10 min at 4 °C, followed by extraction of DNA from both pellet and supernatant fractions using magnetic sorbents.

PCR amplification

PCR was carried out using a mixture containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 0.2 mM dNTPs, 1.6 mM Mg^{2+}, 250 nM of each primer, 2 units of Taq DNA polymerase for low weight (1841 base pairs and lower) fragments and a Taq/Pfu DNA polymerase mix for high-weight (larger than 1841 base pairs) fragments. For amplification of mitochondrial DNA fragments, the following primers were used: MCF1 5′-CCACTCATTCATTGACCTACCTGCC-3′ and DlRev 5′-TAGGTGATTGGGTTTTGCGGACTA-3′ for a 1841 bp product; MC2F 5′-CCAGCATTCCAGTCCTCACAATACC-3′ and MC2R 5′-GCGGCAATATATAGTTGTGCTACT-TG-3′ for 483 b.p product; mmit1f 5′-GCCAGCCTGACCCATAGCCATAATAT-3′ and 5′-GAGAGATTTTATGGGTGTAATGCGG-3′ for 10090 bp product; MAF 5′-CGACAGCTAAGACCCAAACTGGG-3′ and MAR 5′-CCCATTTCTTCCCATTTCATTGGC-3′ for a 316-bp product. Aliquots of each reaction were amplified using a thermocycler with the following cycle profile. 1841 bp and lower fragments – 95 °C for 5 min; 28 cycles: 95 °C for 1 min (denaturation), 60 °C for 1 min (annealing), and 72 °C for 2 min (elongation), followed by 72 °C for 5 min; and kept at 4 °C. 10,090 bp 95 °C for 5 min; 30 cycles: 95 °C for 1 min (denaturation), 65 °C for 1 min (annealing and elongation), followed by 72 °C for 5 min; and kept at 4 °C. The amplified fragments were analyzed electrophoretically using 0.8% (10,090-bp fragment) and 1.2% (1841-bp and smaller fragments) agarose gel with ethidium bromide.

Results and discussion

Addition of calcium to mitochondria in the presence of inorganic phosphate induced a time-dependent decrease in $\Delta\psi$ (fig.1, trace 2) and mitochondrial swelling (not shown). A potent inhibitor of MPT, CsA, which was added to mitochondria prior to calcium (trace 3 vs trace 2) completely prevented the swelling and the drop in $\Delta\psi$, indicating that these changes involved MPT pore opening. Control mitochondria (trace 1) and mitochondria incubated with CsA (trace 3) maintained $\Delta\psi$ until FCCP, an uncoupler of oxidative phosphorylation, was added.

After induction of the MPT, mitochondria were separated from the buffer by centrifugation at 12,500 g for 10 min at 4 °C and the content of mtDNA released from mitochondria into the supernatant was analyzed. Control mitochondria were incubated for the same time interval without Ca^{2+}. mtDNA was extracted from the supernatants using magnetic sorbent, dissolved in 500 µl of Tris-EDTA buffer, and then amplified by PCR with mtDNA-specific primers.

The amplified fragments of mtDNA are shown in figure 2A. In the serial dilution of DNA released from mi-

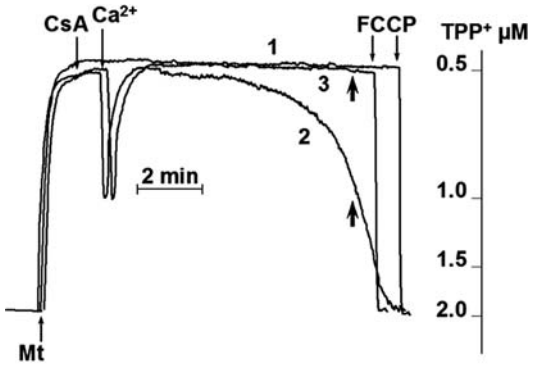

Figure 1. Induction of MPT. Isolated liver mitochondria (1 mg/ml of protein) were suspended in the incubation buffer as described in Materials and methods. Trace 1, control; traces 2 and 3, after a 2 min stabilization period, mitochondria were loaded with 50 nmol Ca^{2+}. Trace 3, 1 µM CsA was added before addition of 50 nmol Ca^{2+}. Mitochondrial samples for mtDNA release studies were withdrawn where indicated (thick arrows).

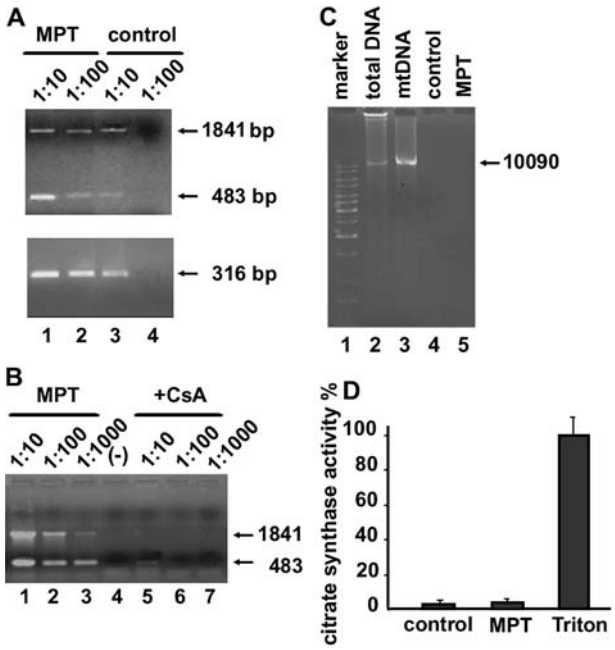

Figure 2. mtDNA fragments released after induction of MPT. (*A*) Lanes 1 and 2, DNA obtained from the supernatant after MPT induction; lanes 3 and 4, DNA from the supernatant of mitochondria incubated in non-MPT conditions. (*B*) Lane 1, marker; lane 2, total DNA isolated from the liver homogenate; lane 3, mitochondrial DNA; lane 4, large fragments of DNA from control mitochondria; lane 5, mitochondria that underwent MPT. (*C*) Effect of CsA on the release of mtDNA fragments. Lanes 1–3, DNA obtained from the supernatant after MPT induction; lanes 5–7, DNA from the supernatant of mitochondria incubated in the presence of CsA; lane 4, negative control (no DNA). (*D*) Release of citrate synthase after MPT induction and treatment with 0.05% Triton X-100. MPT was induced as described in figure 1.

tochondria after MPT induction, the amplification showed a positive signal in all dilutions (lanes 1 and 2), whereas for control mitochondria, the signal was weaker and detectable only after 1:10 dilution (lane 3). Similar results were obtained for all the fragments tested – 316, 483 and 1841 bp. This clearly demonstrates that MPT pore opening can trigger the release of mtDNA fragments from mitochondria. The amount of mtDNA fragments released upon MPT induction decreased with the size of the fragments: large fragments (10,090 bp) were not detected (fig.2B). To prove that the release of mitochondrial fragments was mediated by pore opening, mtDNA was also extracted from the supernatant obtained after removal of mitochondria incubated in the presence of CsA.

Inhibition of MPT induction by CsA (fig. 1, trace 3) completely prevented the release of mtDNA fragments (fig. 2C). As can be seen, in the serial dilution of DNA released from mitochondria that underwent MPT, the amplification showed a positive signal at all dilutions, whereas for mitochondria incubated in the presence of CsA, the signal was much weaker and detectable only after 1:10 dilution. No amplification was obtained for the negative control sample (without DNA). Comparison of lanes 3 (fig. 2A) and 5 (fig. 2C) shows that control mitochondria released a slight amount of mtDNA, whereas mitochondria treated with CsA did not. This can likely be explained by the inherent heterogeneity of mitochondria in terms of their sensitivity to calcium [11]. In the control sample, a small subpopulation of 'weak' mitochondria may undergo the permeability transition stimulated by a trace amount of calcium in the incubation buffer and hence release mtDNA fragments; but in the presence of CsA this possibility is completely excluded.

To ensure that the fragments of mtDNA detected in the supernatant after MPT induction were not a consequence of mitochondrial lysis, the activity of citrate synthase, a matrix enzyme, in the supernatant after MPT induction was examined. The activity of citrate synthase after treatment of mitochondria with a detergent, Triton X-100, was used as 100%. Figure 2D shows that induction of the MPT does not affect retention of matrix proteins; therefore, the release of mtDNA fragments was not due to the loss of inner mitochondrial membrane integrity.

However, the size of the pore is known allow passage of molecules smaller than 1.5 kDa. Undoubtedly, the molecular weight of released fragments was markedly higher, but since the diameter of the pore is around 3 nm, this might be sufficient for the passage of linear mtDNA fragments whose diameter is about 2 nm.

The physiological (as well as pathological) significance of pore opening became a subject of vigorous discussions soon after the phenomenon was discovered. Transient pore opening was considered a safety device responsible for the release of accumulated Ca^{2+} and could thereby be involved in Ca^{2+} signaling and propagation of 'Ca^{2+}

waves' in the cytoplasm [12]. According to another point of view, the opening of the pore and subsequent uncoupling of mitochondria could have inhibit the production of ROS [13] and therefore protect cells from oxidative stress. The MPT was originally believed to be the root mechanism responsible for cytochrome c release in response to various stimuli inducing apoptotic cell death [14]; however, more recently, this notion has been challenged. Ample evidence from more recent studies suggests that pro-apoptotic members of Bcl-2 family proteins (such as Bid, Bax, Bak) stimulate release of cytochrome c and other proteins from the intermembrane space of mitochondria without disturbing mitochondrial integrity and functional activity [15].

Opening of the MPT pore was clearly shown to be the key event in ischemia/reperfusion-induced tissue damage [16]. Indeed, the MPT leads to a severe impairment of mitochondrial functional activity, including the uncoupling of oxidative phosphorylation and a drop in $\Delta\psi$. Uncoupling of oxidative phosphorylation will cause mitochondrial ATPase to hydrolyze ATP, rather than produce it. As a result, the intracellular concentration of ATP will drop, leading to the disruption of Ca^{2+} homeostasis, activation of catabolic enzymes and, finally, to necrotic cell death [16]. Our results show for the first time that opening of the MPT pore can trigger the release of DNA fragments from mitochondria. Given that transient opening of the pore in a certain subpopulation of mitochondria may occur even under normal physiological conditions, the release of mtDNA fragments and their subsequent incorporation into nuclear DNA may occur continuously. In pathological situations, the probability of pore opening as well as the release of mtDNA fragments increases considerably. This might have harmful consequences for the cell since this is one of the sources of genomic instability.

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