A folding variant of human α-lactalbumin induces mitochondrial permeability transition in isolated mitochondria

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A human milk fraction containing multimeric α-lactalbumin (MAL) is able to kill cells via apoptosis. MAL is a protein complex of a folding variant of α-lactalbumin and lipids. Previous results have shown that upon treatment of transformed cells, MAL localizes to the mitochondria and cytochrome c is released into the cytosol. This is followed by activation of the caspase cascade. In this study, we further investigated the involvement of mitochondria in apoptosis induced by the folding variant of α-lactalbumin. Addition of MAL to isolated rat liver mitochondria induced a loss of the mitochondrial membrane potential (ΔΨm), mitochondrial swelling and the release of cytochrome c. These changes were Ca2+ dependent and were prevented by cyclosporin A, an inhibitor of mitochondrial permeability transition. MAL also increased the rate of state 4 respiration in isolated mitochondria by exerting an uncoupling effect. This effect was due to the presence of fatty acids in the MAL complex because it was abolished completely by BSA. BSA delayed, but failed to prevent, mitochondrial swelling as well as dissipation of ΔΨm, indicating that the fatty acid content of MAL facilitated, rather than caused, these effects. Similar results were obtained with HAMLET (human α-lactalbumin made lethal to tumour cells), which is native α-lactalbumin converted in vitro to the apoptosis-inducing folding variant of the protein in complex with oleic acid. Our findings demonstrate that a folding variant of α-lactalbumin induces mitochondrial permeability transition with subsequent cytochrome c release, which in transformed cells may lead to activation of the caspase cascade and apoptotic death.

Keywords: α-lactalbumin; cytochrome c; mitochondria; mitochondrial permeability transition; tumour cells.
To further support this, we recently showed that HAMLET (human α-lactalbumin made lethal to tumour cells), which is native α-lactalbumin converted in vitro into the molten globule-like state, was as effective as MAL at inducing apoptosis [9]. Both MAL and HAMLET contain fatty acids, which are believed to be important for the stabilization of the molten globule-like folding state of the protein. In order to investigate the role of mitochondria in apoptosis triggered by this folding variant of α-lactalbumin, the effects of MAL and HAMLET were examined using isolated rat liver mitochondria as a model system. Here we show that MAL, as well as HAMLET, induces MPT in isolated mitochondria resulting in a loss of ΔΨm, mitochondrial swelling and release of proteins from the mitochondrial intermembrane space. We have also shown that the fatty acids in the α-lactalbumin complexes facilitated MPT induction by mild uncoupling of mitochondria.

MATERIALS AND METHODS

Preparation of mitochondria

Liver mitochondria were isolated from male Wistar rats (150–200 g) by differential centrifugation in buffer containing 220 mM mannitol, 70 mM sucrose, 5 mM Hepes and 1 mM EDTA, pH 7.4 (MSH) [10]. The mitochondrial fraction was washed and resuspended (90–100 mg protein·mL−1) in MSH buffer without EDTA. The protein concentration was determined using the BCA protein assay (Pierce, Rockford, IL, USA) with BSA as a standard.

Mitochondrial swelling

Swelling of isolated rat liver mitochondria was estimated spectrophotometrically as a decrease in absorbance measured at 540 nm [11]. Mitochondria (0.5 mg protein·mL−1) were suspended in buffer A (0.25 M sucrose, 10 mM Tris/Mops, 2.5 mM KH2PO4, 5 mM succinate and 2 μM rotenone, pH 7.4) and pre-incubated in a thermostated cuvette (30 °C) in the presence of 20 nmoles of Ca2+ per mg of protein. Mitochondrial swelling was triggered by different concentrations of MAL or HAMLET. Mitochondrial swelling was also assessed in the presence of inhibitors [1 μM CsA, 50 μM ubiquinone 0 (Ub0), 10 μM ruthenium red (RuRed)] as well as in the presence of 50 μM EGTA or 0.05% BSA.

Release of intermembrane proteins from isolated mitochondria

Mitochondrial swelling was induced as described above and 5 min later mitochondria were pelleted by centrifugation (10 000 g 15 min). The post-mitochondrial supernatants were concentrated ≈ 10-fold using a vacuum lyophilizer, diluted with 4 × Laemmli’s loading buffer without glycerol and subjected to Western blot analysis. Briefly, samples were boiled for 4 min and the proteins were separated by electrophoresis on 15% SDS/polyacrylamide gels. The proteins were then electroblotted onto a nitrocellulose membrane, 0.45 μm (Sartorius AG, Göttingen, Germany), blocked overnight in a high-salt buffer (50 mM Tris/base, 500 mM NaCl, pH 7.4) containing 5% dried milk and 1% BSA, and probed with antibodies against cytochrome c (1 : 2500) or adenylate kinase-2 (1 : 2500). Primary antibody binding was detected using secondary goat antimouse (1 : 10 000) or a goat antirabbit (1 : 10 000) conjugated with horseradish peroxidase and visualized by enhanced chemiluminescence as described in the manufacturer’s instructions (Amersham, Buckinghamshire, UK).

Loss of ΔΨm in isolated mitochondria

Estimation of ΔΨm in isolated mitochondria was performed using an electrode sensitive to the cation tetraphenylphosphonium (TPP+). Energized mitochondria rapidly accumulate TPP+ from the incubation buffer compatible with the formation of a membrane potential. Mitochondria (1.5 mg protein·mL−1) were incubated at 25 °C in 2 mL of a buffer containing 125 mM KCl, 2.5 mM KH2PO4, 5 mM Tris, 5 mM succinate and 2.5 μM TPP+, pH 7.5. Rotenone (2 μM) was added immediately after the addition of mitochondria to the incubation medium. Mitochondria were loaded with 40 nmoles of Ca2+ per mg of protein and the loss of ΔΨm was monitored upon addition of MAL or HAMLET in the absence or presence of 0.05% BSA, 1 μM CsA or 500 μM ATP.

Mitochondrial respiration

Oxygen consumption in isolated rat liver mitochondria was measured using a Clark-type oxygen electrode (Yellow Spring Instrument Co., OH, USA) at 25 °C. The incubations contained 1.5 mg protein·mL−1 in a buffer consisting of 125 mM KCl, 5 mM Tris, 2.5 mM KH2PO4, pH 7.4. Mitochondria were energized with 5 mM succinate in the presence of 5 μM rotenone. State 3 respiration was then initiated by the addition of 500 nmol ADP. The rate of uncoupled respiration was determined after addition of 3 μM carbonyl cyanide m-chlorophenylhydrazone (CCCP) to the incubation mixture. The effect of different concentrations of MAL and oleic acid (dissolved in ethanol) on oxygen consumption was determined in the absence or presence of 0.05% BSA. Mitochondria with a respiritory control ratio (defined as the rate of respiration in the presence of ADP divided by the rate obtained following the expenditure of ADP) > 4 were used in the experiments.

RESULTS

MAL-induced swelling of Ca2+-loaded rat liver mitochondria and release of intermembrane space proteins

Within minutes of adding MAL to Ca2+-loaded mitochondria, large amplitude swelling was induced that was concentration dependent (Fig. 1A). In our experiments, the concentration of Ca2+ was not high enough to induce swelling in the absence of MAL. However, the presence of Ca2+ was obligatory, because EGTA, a calcium chelator, and RuRed, an inhibitor of mitochondrial Ca2+ uptake, prevented MAL-induced swelling (Fig. 1B). Inhibitors of the MPT pore, such as CsA and Ub0 [12], also completely prevented the swelling of mitochondria (Fig. 1B), indicating that MAL-induced swelling was associated with MPT induction.

Mitochondrial swelling was accompanied by the release of cytochrome c and adenylate kinase-2 (AK-2) from the mitochondrial intermembrane space (Fig. 1C). This release was inhibited by CsA, Ub0, EGTA and RuRed, suggesting that MPT was required for the release of these proteins from the mitochondria.

Loss of ΔΨm induced by MAL in isolated mitochondria

Addition of MAL to Ca2+-loaded mitochondria caused a biphasic decrease in ΔΨm (Fig. 1D). The first rapid phase was
followed by a larger, time-dependent second phase of membrane depolarization that resulted in a complete collapse of Δψm. Inhibitors of MPT, such as CsA and ATP, blocked the second phase of the decrease in Δψm, but failed to prevent the initial shift. The first phase of the decrease in Δψm was also observed when mitochondria were incubated with MAL in the absence of Ca2+ (data not shown). These data suggested that, in contrast to the second phase, the first decrease in Δψm was not a consequence of MPT.

Stimulation of state 4 respiration induced by MAL
In the experiments described above, Δψm was supported by the mitochondrial respiratory chain via oxidation of succinate. The CsA-insensitive decrease in Δψm upon addition of MAL could be due to either inhibition of respiration or uncoupling of mitochondria. In order to investigate this, mitochondrial oxygen consumption was measured. Addition of MAL to mitochondria accelerated state 4 respiration in a concentration-dependent manner (Fig. 2A). This resulted in a decrease in the respiration control ratio, which was more prominent at higher concentrations of MAL. An increase in the rate of state 4 respiration indicated that MAL enhanced the proton permeability of the inner mitochondrial membrane and thereby induced mild uncoupling of mitochondria. MAL caused only a slight decrease in the rate of respiration in the presence of ADP (state 3) or the protonophore CCCP, suggesting that neither mitochondrial ATPase nor the components of the respiratory chain were affected. Acceleration of respiration was not a consequence of MPT, because it occurred in the absence of Ca2+ and was not prevented by CsA (data not shown).

The involvement of fatty acids in MAL-induced effects on respiration, loss of Δψm and mitochondrial swelling
Mild uncoupling of mitochondria may be due to the fatty acid content of MAL. Lipid extraction of MAL yielded ≈5–10% (w/w) lipids (unpublished data). Oleic acid (18 : 1) is the most abundant fatty acid in human milk [13] and also the major fatty acid in MAL. Pre-incubation of mitochondria with BSA, which sequesters fatty acids, prevented the increase in state 4 respiration as well as the decrease in the respiration control ratio induced by MAL (Fig. 2B). Addition of the corresponding amount of oleic acid to mitochondria induced a BSA-inhibitable increase in state 4 respiration and a decrease in the respiration control ratio (Fig. 2B). These results indicated that the uncoupling of respiration was due to fatty acids present in MAL.
The effect of BSA on MAL-induced swelling and loss of $\Delta \Psi_m$ in isolated mitochondria is illustrated in Fig. 3. BSA prevented the first decline in $\Delta \Psi_m$ upon addition of MAL (Fig. 3B), which was insensitive to CsA, indicating that the initial decrease in $\Delta \Psi_m$ was due to partial uncoupling of mitochondria by fatty acids. However, MAL was still able to induce swelling of mitochondria and collapse $\Delta \Psi_m$ in the presence of BSA, although BSA delayed these events slightly (Fig. 3A).

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Induction of MPT by HAMLET

HAMLET is prepared in vitro by subjecting EDTA-treated $\alpha$-lactalbumin to ion-exchange chromatography on a matrix conditioned with oleic acid [9]. In order to investigate whether HAMLET had similar effects as MAL on isolated mitochondria, the MPT-inducing capacity of HAMLET was examined. HAMLET preparations contain low amounts of EDTA ($\approx 10 \mu$M), which is known to suppress the induction of MPT by binding Ca$^{2+}$ in the incubation buffer. Taking this into consideration, experiments with HAMLET were performed in the presence of 1 mM Mg$^{2+}$, which saturated EDTA and overcame its effect on MPT induction. Similar to MAL, HAMLET induced swelling and biphasic dissipation of $\Delta \Psi_m$, which resulted in the release of proteins from the intermembrane space (Fig. 4). These events were a consequence of MPT induction because CsA, Ub0 and RuRed inhibited swelling and the second phase decrease in $\Delta \Psi_m$. HAMLET also exhibited similar effects on mitochondrial respiration as MAL, and BSA abolished its uncoupling effect (data not shown). BSA delayed, but did not abolish, the HAMLET-induced decrease in $\Delta \Psi_m$, again demonstrating that the fatty acids present in the $\alpha$-lactalbumin complexes were not responsible for opening of the MPT pore.

**DISCUSSION**

A previous study reported that cytochrome c release and caspase activation are involved in MAL-induced apoptosis in transformed cells [8]. MAL was internalized into the cytoplasm and co-localized with mitochondria. The results presented in this study show that MAL may induce apoptosis via a direct interaction with mitochondria, resulting in an opening of the MPT pore with the subsequent release of mitochondrial constituents, including cytochrome c, from the intermembrane space.

Although the precise mechanism of release of cytochrome c during apoptosis is unclear, in many experimental models this event is associated with a collapse of $\Delta \Psi_m$ due to Ca$^{2+}$-dependent MPT pore opening [14]. Opening of the MPT pores renders the mitochondrial inner membrane nonselectively permeable to small molecules ($< 1.5$ kDa). As a consequence, water and solutes enter the mitochondrial matrix via osmosis, which leads to swelling and rupture of the outer mitochondrial membrane. Although Ca$^{2+}$ is the obligatory pore activator, oxidative stress, adenine nucleotide depletion and a decrease in...
Fig. 4. HAMLET induces MPT in isolated mitochondria. (A) Rat liver mitochondria (0.5 mg·mL⁻¹) were pre-incubated with 20 nmol of Ca²⁺ per mg of protein at 30 °C. Mitochondrial swelling was induced by addition of HAMLET (20 μg·mg protein⁻¹) and monitored continuously as a change in ΔΨₘ. Curve a: control; curve b: HAMLET; curves c-e: HAMLET and 1 μM CsA; 50 μM Ub₀; and 10 μM RuRed, respectively. (B) HAMLET-induced release of cytochrome c from isolated mitochondria. Mitochondria (0.5 mg·mL⁻¹) were pre-incubated with 20 nmol of Ca²⁺ per mg of protein at 30 °C. After 5 min incubation, untreated or HAMLET-treated (20 μg·mg protein⁻¹) mitochondria were pelleted and the supernatants were subjected to Western blot analysis as described in Materials and methods. The membrane was probed with an antibody against cytochrome c. (C) Effect of BSA and CsA on HAMLET-induced loss of ΔΨₘ. Mitochondria (1.5 mg·mL⁻¹) were loaded with Ca²⁺ (40 nmol-mg protein⁻¹) and the decrease in ΔΨₘ was induced by addition of HAMLET (60 μg·mg protein⁻¹). Curve a: control; curve b: HAMLET; curve c: HAMLET and 0.05% BSA; curve d: HAMLET and 1 μM CsA; curve e: 0.05% BSA.

ΔΨₘ can significantly increase the sensitivity of MPT to Ca²⁺ [15].

As mentioned above, MAL is a fraction of human milk containing a complex of a folding variant of α-lactalbumin and lipids. In contrast, HAMLET is not a fraction of human milk, but native α-lactalbumin converted in vitro to the apoptosis-inducing folding variant of the protein. While native α-lactalbumin is unable to induce apoptosis (or MPT when added to isolated mitochondria), HAMLET and MAL kill tumour cells via apoptosis with similar kinetics.

MAL added to isolated rat liver mitochondria induced swelling of mitochondria, followed by the release of cytochrome c and AK-2 into the medium. The MPT inhibitors CsA and Ub₀ abolished this release. CsA is a classical inhibitor of MPT, while Ub₀ was recently shown to interact with the MPT pore and inhibit its opening [12]. Both swelling and the release of cytochrome c and AK-2 from MAL-treated mitochondria were prevented by chelation of Ca²⁺, or inhibition of Ca²⁺ entry into mitochondria by RuRed, suggesting that these events are Ca²⁺-dependent. Similar results were obtained with HAMLET, further supporting the concept that the folding variant of α-lactalbumin is the active component in MAL.

Investigation of the effect of the folding variant of α-lactalbumin on mitochondria revealed that, in addition to MPT induction, this complex also affected mitochondrial respiration. Both MAL and HAMLET induced mild uncoupling of mitochondria, which resulted in a concentration-dependent acceleration of mitochondrial respiration (state 4) and a marked decrease in the respiration control ratio. It should be stressed that alteration of mitochondrial respiration occurred without Ca²⁺ loading and was not prevented by CsA, hence MPT was not involved. However, the activation of state 4 respiration and decrease in respiration control ratio values were completely restored by BSA.

The dual effects of the folding variant of α-lactalbumin became even more evident when estimating the changes in ΔΨₘ. Addition of this complex to Ca²⁺-loaded mitochondria induced a concentration-dependent rapid shift in ΔΨₘ followed by a time-dependent collapse of ΔΨₘ. CsA, as well as ATP, prevented the second and more severe phase of decreased ΔΨₘ, but not the first shift, which was apparently brought about by fatty acids because it was prevented completely by BSA. However, BSA failed to rescue the mitochondria from MPT and was only able to delay the collapse of ΔΨₘ (as well as swelling), suggesting that fatty acids in the α-lactalbumin complexes sensitize mitochondria towards permeability transition.

The lipid component of MAL and HAMLET is believed to be important for maintaining α-lactalbumin in the active apoptosis-inducing folding state, the so-called molten globule-like state [6], which is an intermediate state between the native and denatured form of the protein. This folding variant of α-lactalbumin has a native-like secondary structure, but a less well-defined tertiary structure.

The ability of free fatty acids, such as oleic acid and palmitic acid, to uncouple mitochondria, and thereby cause an increased rate of respiration, has been well documented [16]. In our experiments, the addition of a corresponding amount of oleic acid to mitochondria induced similar effects as MAL and HAMLET on both state 4 respiration and respiration control ratio in a BSA-inhibitable manner. When added to Ca²⁺-loaded mitochondria, the fatty acid itself induced a rapid partial decrease in ΔΨₘ (data not shown) but not the secondary collapse seen upon addition of MAL or HAMLET; hence, oleic acid did not induce opening of the MPT pore under our experimental conditions. The fatty acids in both MAL and HAMLET only facilitated pore opening, which is in agreement with other studies showing that a partial uncoupling of mitochondria favours MPT induction [17,18].
In conclusion, our results demonstrate that a folding variant of α-lactalbumin induces MPT in isolated mitochondria. Although the relevance of this finding for MAL/HAMLET-induced apoptosis requires further investigation, it seems likely that the MPT-mediated mitochondrial pathway contributes to caspase activation and apoptosis also in intact cells treated with these agents.

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