Activation of the potato plant uncoupling mitochondrial protein inhibits reactive oxygen species generation by the respiratory chain

Alicia J. Kowaltowski, Alexandre D.T. Costa, Anibal E. Vercesi*

Departamento de Patologia Clínica, Faculdade de Ciências Médicas, Universidade Estadual de Campinas, 13083-970 Campinas, SP, Brazil

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Abstract A variety of plant tissues contain an uncoupling mitochondrial protein (PUMP), recently described and characterized by our group. In this study we show that the inhibition of PUMP activity in potato tuber mitochondria significantly increases mitochondrial H_2O_2 generation, while PUMP substrates, such as linoleic acid, reduce mitochondrial H_2O_2 generation. This H_2O_2 generation occurred mainly by the dismutation of superoxide radicals formed through monoelectronic reduction of O_2 by semiquinone forms of coenzyme Q. The results presented suggest that protection against mitochondrial oxidative stress may be a physiological role of PUMP.

Key words: Reactive oxygen species; Free radical; Plant uncoupling mitochondrial protein; Coenzyme Q; Mitochondrion

1. Introduction

The generation of reactive oxygen species (ROS) by the mitochondrial respiratory chain is a physiological and continuous process. Indeed, up to 2% of the oxygen consumed by the mitochondrial respiratory chain suffers monoelectronic reduction, leading to the production of superoxide radicals [1–3]. Under physiological conditions, ROS are readily removed by mitochondrial antioxidant systems, but under pathological conditions, in which these antioxidant systems are defective or mitochondrial ROS generation is increased, oxidative damage of the inner mitochondrial membrane may occur, with impairment of mitochondrial function (for review see [4]). In this situation, accidental or programmed cell death may follow mitochondrial dysfunction [5].

One of the many proposed mechanisms which mitochondria use to decrease ROS generation is the uncoupling between mitochondrial respiration and membrane potential, resulting in increased respiratory rates (for review see [6,7]). Protonophores are well known to decrease mitochondrial ROS generation [3], and mitochondrial respiratory energy dissipative pathways, such as the plant alternative oxidase [8,9] and mammalian uncoupling proteins [10], have been demonstrated to present the same effect. Based on these observations, Skulachev [6,7] formulated the hypothesis that mild uncoupling of mitochondria may be an effective mechanism to reduce mitochondrial ROS generation without seriously compromising cellular energetics.

*Corresponding author. Fax: (55) (19) 2393184.
E-mail: anibal@obelix.unicamp.br

We have recently described and characterized a plant uncoupling mitochondrial protein (PUMP) [11–14], present in a variety of plant tissues (P. Ježek, A.D.T. Costa, P. Arruda and A.E. Vercesi, unpublished results). Later, the complementary DNA from potatoes that probably encodes PUMP was identified and expressed in yeast, resulting in mitochondrial membrane potential decrease in these cells [15]. PUMP is a 32 kDa protein which transports anionic fatty acids across the inner mitochondrial membrane [12], in a manner similar to the mammalian brown adipose tissue uncoupling protein [16,17]. Protonated fatty acids are capable of diffusing through the inner mitochondrial membrane from the mitochondrial intermembrane space to the matrix [17]. PUMP removes the anionic fatty acids from the mitochondrial matrix back into the intermembrane space, resulting in fatty acid cycling, and mitochondrial uncoupling [12]. In consequence, the addition of anionic fatty acids, such as linoleic acid, to plant mitochondria results in mitochondrial uncoupling, while the presence of ATP, which inhibits PUMP, and bovine serum albumin (BSA), which removes free fatty acids, increases the membrane potential of these mitochondria [11–14].

In this report we demonstrate that the activity of PUMP decreases mitochondrial reactive oxygen species formation at the respiratory chain, in a manner dependent on the decrease in mitochondrial membrane potential induced by PUMP activity.

2. Materials and methods

2.1. Isolation of rat liver and potato tuber mitochondria

Mitochondria were isolated by conventional differential centrifugation, as described [11,12,18].

2.2. Standard incubation procedure

The experiments were carried out at 28°C, with continuous magnetic stirring, in a standard reaction medium containing 125 mM sucrose, 65 mM KCl, 10 mM HEPES buffer pH 7.2, 4 μM rotenone, 1 μM horseradish peroxidase, 1 μM scopoletin and 1 μM cyclosporin A. Other additions are indicated in the figure legends. The results shown are representative of a series of at least three experiments.

2.3. Determination of mitochondrial H_2O_2 generation

H_2O_2 production was assessed by the oxidation of scopoletin by horseradish peroxidase in the presence of H_2O_2 [19]. Scopoletin fluorescence was monitored at excitation and emission wavelengths of, respectively, 365 and 450 nm, on a Hitachi F-4010 fluorimeter. Calibration was performed by adding known quantities of H_2O_2. Cyclosporin A was present in all determinations in order to prevent artifacts due to mitochondrial swelling.

2.4. Measurements of mitochondrial transmembrane electrical potential ($\Delta\Psi$)

Mitochondrial $\Delta\Psi$ was estimated through fluorescence changes of safranine O (5 μM), recorded on a model F-4010 Hitachi spectrofluorometer (Hitachi, Ltd., Tokyo, Japan) operating at excitation and emission wavelengths of 495 and 586 nm, with a slit width of 5 nm.

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2.5. Determination of mitochondrial Ca^{2+} uptake

Mitochondrial Ca^{2+} uptake was determined by measuring the absorbance changes of arsenazo III on a SLM Aminco DW2000 spectrophotometer at the wavelength pair of 675/685 nm.

2.6. Materials

Horseradish peroxidase (type IV-A), scopoletin, safranine O, H_2O_2, cyclosporin A, HEPES, rotenone, antimycin A, myxothiazol, linoleic acid, phenylvaleric acid, heptylbenzoic acid, BSA, ATP, carbonyl cyanide *m*-chlorophenylhydrazone (FCCP) and succinate were obtained from Sigma Chemical Company (St. Louis, MO, USA). All other reagents were commercial products of the highest purity grade available.

3. Results and discussion

We have previously shown that the addition of linoleic acid to respiring potato mitochondria reduces their membrane potential, while full coupling of these mitochondria was obtained only in the presence of ATP and BSA [11,12]. These results are reproduced in Fig. 1, which demonstrates that the membrane potential of potato mitochondria respiring on succinate (state 4, panel A, line b) is increased by the presence of ATP and BSA (line a) and reduced by the presence of linoleic acid (line c). These effects were attributed to the presence of a plant mitochondrial uncoupling protein (PUMP) similar to the mammalian mitochondrial uncoupling protein [16,20], which is inhibited by purine nucleotides and uses fatty acids as substrates [11–14]. Rat liver mitochondria, which do not contain uncoupling proteins [10], did not suffer significant alterations in membrane potential (Fig. 1B, line b) in the presence of ATP plus BSA (line a) or linoleic acid (line c).

Fig. 2 monitors mitochondrial generation of H_2O_2 through the oxidation of scopoletin in the presence of horseradish peroxidase. We observed that the generation of H_2O_2 by potato mitochondria incubated in the presence of rotenone (panel A, line d) is largely increased by the addition of succinate (line c). The addition of the PUMP substrate linoleic acid significantly decreased potato mitochondrial respiration-supported H_2O_2 generation (line e) while phenylvaleric acid, a fatty acid unable to flip-flop across lipid bilayers, which does not act as a PUMP substrate [13], did not inhibit mitochondrial H_2O_2 generation in the presence of succinate (line b). The addition of ATP, which inhibits PUMP activity, and

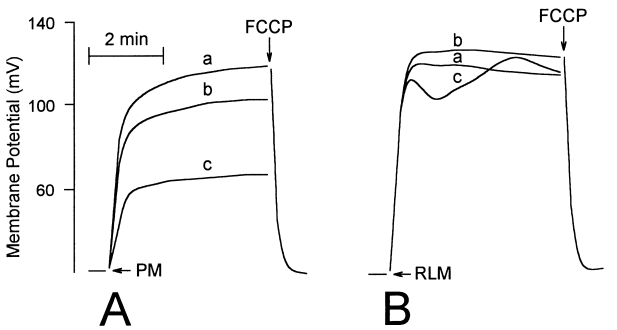

Fig. 1. Effect of PUMP substrates and inhibitors on mitochondrial membrane potential. Potato mitochondria (PM, 1 mg/ml, A) or rat liver mitochondria (RLM, 1 mg/ml, B) were incubated in standard reaction medium in the absence of scopoletin and horseradish peroxidase and in the presence of 5 μM safranine O, 2 mM succinate, 100 μM EGTA, 1 μM oligomycin and: (lines a) 0.1% BSA plus 2.5 mM ATP, (lines b) no further additions or (lines c) 40 μM linoleic acid. 5 μM FCCP was added to all experiments where indicated.

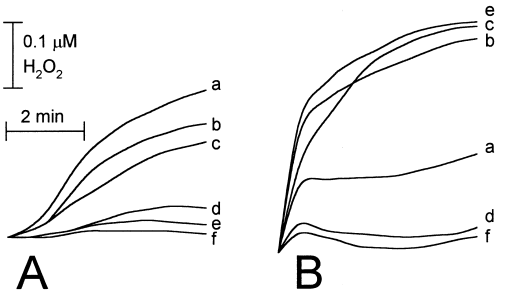

Fig. 2. Effect of PUMP substrates and inhibitors on mitochondrial respiration-stimulated H_2O_2 generation. Potato mitochondria (0.5 mg/ml, A) or rat liver mitochondria (0.5 mg/ml, B) were incubated in standard reaction medium containing 2 mM succinate and: (lines a) 0.1% BSA plus 2.5 mM ATP, (lines b) 40 μM phenylvaleric acid, (lines c) no further additions, (lines e) 40 μM linoleic acid or (lines f) 1 μM FCCP. Lines d represent experiments conducted in the absence of succinate.

BSA, which removes free fatty acids from the mitochondrial suspension, increased mitochondrial H_2O_2 generation (line a), confirming that PUMP activity decreases mitochondrial ROS generation. The presence of the uncoupler FCCP completely inhibited mitochondrial H_2O_2 generation both in the presence (not shown) and in the absence (line f) of BSA and ATP, suggesting that the increase in mitochondrial H_2O_2 generation induced by PUMP inhibition is dependent on the effects of PUMP on mitochondrial membrane potential. This increase in mitochondrial H_2O_2 generation may be attributed directly to a decrease in mitochondrial membrane potential or to the increased rates of mitochondrial respiration under lower membrane potentials.

Parenchymal rat liver cells do not contain uncoupling proteins, such as the uncoupling protein-2, distributed in many mammalian tissues [10]. Fig. 2 shows that the H_2O_2 generation of mitochondria isolated from rat liver incubated in the presence of rotenone (panel B, line d), was also largely increased by the addition of succinate (line c). The presence of linoleic acid did not decrease this production (line e), which was only reduced in the presence of FCCP (line f). The addition of BSA and ATP to rat liver mitochondria partially inhibited mitochondrial H_2O_2 generation (line a), probably due to the decrease in the free Ca^{2+} concentration of the reaction medium promoted by ATP and BSA, which complex Ca^{2+}. Ca^{2+} is well known to increase mitochondrial ROS generation (for review see [4]). BSA may also quench mitochondrial-generated ROS.

In Fig. 3, mitochondrial H_2O_2 generation was stimulated by the addition of Ca^{2+} to a mitochondrial suspension respiring on succinate. Potato mitochondria, which are much less active in Ca^{2+} accumulation than liver mitochondria (results not shown), required the addition of high Ca^{2+} concentrations (500 μM) in order to produce a burst in mitochondrial ROS generation (panel A, line b) similar to that obtained by the addition of 50 μM Ca^{2+} to rat liver mitochondria (panel B, line b). Like respiration-induced mitochondrial H_2O_2 generation in potato mitochondria, the Ca^{2+}-induced burst in H_2O_2 production was decreased by the presence of linoleic acid (panel A, line d), but not by phenylvaleric acid (line c), and was increased by the presence of ATP plus BSA (line a). Rat liver mitochondrial H_2O_2 production stimulated by Ca^{2+} (panel B, line b) was not affected by the presence of linoleic

(line d) or phenylvaleric acids (line c), and was partially inhibited by ATP plus BSA (line a). Indeed, the addition of 50 μM Ca^{2+} to the reaction medium in the presence of ATP results in a free Ca^{2+} concentration lower than 10 μM, which would certainly explain the reduction of the Ca^{2+}-stimulated burst in mitochondrial H_2O_2 generation. The presence of FCCP decreased mitochondrial H_2O_2 generation stimulated by Ca^{2+} in both rat liver and potato mitochondria (lines e). This decrease was most evident in rat liver mitochondria, which in the presence of FCCP were unable to take up 50 μM Ca^{2+} down their membrane potential. In potato mitochondria, the addition of 500 μM Ca^{2+} resulted in Ca^{2+} influx even in the presence of FCCP, driven by a chemical Ca^{2+} gradient [18].

Mitochondrial ROS generation at the respiratory chain occurs mainly due to the transfer of electrons from the semiquinone form of coenzyme Q to oxygen, leading to the formation of the superoxide radical, which is readily dismutated by mitochondrial superoxide dismutase to H_2O_2 [1,3,18,21]. In order to verify if this was the main process occurring in our experimental conditions, we tested the effect of different mitochondrial respiratory chain inhibitors on mitochondrial Ca^{2+}-stimulated H_2O_2 generation (Fig. 4). FCCP was present in all determinations in order to prevent the occurrence of different membrane potentials, and mitochondrial H_2O_2 generation was stimulated by the addition of Ca^{2+}. We observed that H_2O_2 generation in both potato (panel A) and rat liver (panel B) mitochondria incubated in the presence of rotenone and treated with Ca^{2+} (lines c) was stimulated by succinate-supported respiration (lines b) and by the presence of antimycin A, which promotes the accumulation of semiquinone forms of coenzyme Q (lines a). Myxothiazol, which prevents the formation of the semiquinone anion [3], inhibited mitochondrial Ca^{2+}-stimulated H_2O_2 generation (lines d), confirming that this generation occurs mainly due to dismutation of superoxide radicals formed through the transfer of electrons from the semiquinone form of coenzyme Q to molecular oxygen.

The results presented in this paper demonstrate that the decrease in mitochondrial membrane potential induced by PUMP activity inhibits mitochondrial generation of ROS at the level of the semiquinone forms of coenzyme Q, as observed previously with the plant alternative oxidase [8,9] and mammalian uncoupling proteins [10]. The mechanism by

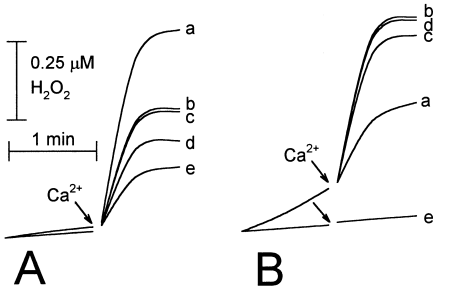

Fig. 3. Effect of PUMP substrates and inhibitors on Ca^{2+}-stimulated mitochondrial H_2O_2 generation. Potato mitochondria (0.5 mg/ml, A) or rat liver mitochondria (0.5 mg/ml, B) were incubated in standard reaction medium containing 2 mM succinate and: (lines a) 0.1% BSA plus 2.5 mM ATP, (lines b) no further additions, (lines c) 40 μM phenylvaleric acid, (lines d) 40 μM linoleic acid or (lines e) 1 μM FCCP. 500 μM Ca^{2+} (A) or 50 μM Ca^{2+} (B) was added where indicated by the arrows.

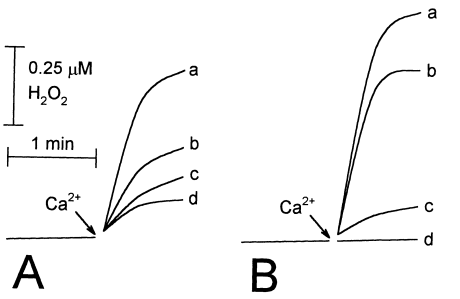

Fig. 4. Effect of respiratory chain inhibitors on Ca^{2+}-stimulated mitochondrial H_2O_2 generation. Potato mitochondria (0.5 mg/ml, A) or rat liver mitochondria (0.5 mg/ml, B) were incubated in standard reaction medium containing 1 μM FCCP and: (lines a) 1 μM antimycin A and 2 mM succinate, (lines b) 2 mM succinate, (lines c) no further additions or (lines d) 1 μg/ml myxothiazol and 2 mM succinate. 500 μM Ca^{2+} was added where indicated by the arrows.

which mitochondrial uncoupling decreases mitochondrial ROS generation is not yet clear, but it may be attributed to the increase in mitochondrial respiration rates, decreasing the life time of the semiquinone forms of coenzyme Q and reducing the probability of superoxide anion formation through electron transfer from semiquinone forms of coenzyme Q to molecular oxygen [7]. Alternatively, as suggested by Liu [2], the presence of low mitochondrial membrane potentials could decrease the reaction between superoxide anions and protons at the cytoplasmic side of the inner mitochondrial membrane, resulting in the formation of the perhydroxyl radical. This would reduce the dismutation of superoxide anions by intramitochondrial superoxide dismutase, decreasing the detection of mitochondrial-generated H_2O_2, because diffusion of the superoxide anions through the inner mitochondrial membrane occurs mainly in the perhydroxyl form [2].

Independently of the mechanism by which mitochondrial uncoupling reduces the generation of ROS at the mitochondrial respiratory chain, the regulation of mitochondrial ROS production should be regarded as a function of respiratory energy dissipative pathways, such as PUMP, the alternative oxidase and mammalian uncoupling proteins. Activation of these pathways promoting a mild uncoupling [7] of mitochondria efficiently reduces mitochondrial ROS generation, and may prevent oxidative mitochondrial damage.

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References

[1] Boveris, A. and Chance, B. (1973) Biochem. J. 134, 707–716.
[2] Liu, S.S. (1997) Biosci. Rep. 17, 259–272.
[3] Turrens, J.F. (1997) Biosci. Rep. 17, 3–8.
[4] Vercesi, A.E., Kowaltowski, A.J., Grijalba, M.T., Meinicke, A.R. and Castilho, R.F. (1997) Biosci. Rep. 17, 43–51.
[5] Zamzami, N., Hirsch, T., Dallaporta, B., Petit, P.X. and Kroemer, G. (1997) J. Bioenerg. Biomembr. 29, 185–193.
[6] Skulachev, V.P. (1996) FEBS Lett. 397, 7–10.
[7] Skulachev, V.P. (1997) Biosci. Rep. 17, 347–366.
[8] Wagner, A.M. and Moore, A.L. (1997) Biosci. Rep. 17, 319–333.

[9] Popov, V.N., Simonian, R.A., Skulachev, V.P. and Starkov, A.A. (1997) FEBS Lett. 415, 87–90.
[10] Nègre-Salvayre, A., Hirtz, C., Carrera, G., Cazenave, R., Troly, M., Salvayre, R., Pénicaud, L. and Casteilla, L. (1997) FASEB J. 11, 809–815.
[11] Vercesi, A.E., Martins, I.S., Silva, M.A.P., Leite, H.M.F., Cuccovia, I.M. and Chaimovich, H. (1995) Nature 375, 24.
[12] Ježek, P., Costa, A.D.T. and Vercesi, A.E. (1996) J. Biol. Chem. 271, 32743–32749.
[13] Ježek, P., Costa, A.D.T. and Vercesi, A.E. (1997) J. Biol. Chem. 272, 24272–24278.
[14] Vercesi, A.E., Chaimovich, H. and Cuccovia, I.M. (1997) Recent Res. Dev. Plant Physiol. 1, 85–91.
[15] Laloi, M., Klein, M., Reismeier, J.W. and Müller-Röber, B. (1997) Nature 389, 135–136.
[16] Garlid, K.D., Orosz, D.E., Modriansky, M., Vassanelli, S. and Ježek, P. (1996) J. Biol. Chem. 271, 2615–2702.
[17] Skulachev, V.P. (1991) FEBS Lett. 294, 158–162.
[18] Kowaltowski, A.J., Castilho, R.F. and Vercesi, A.E. (1995) Am. J. Physiol. 269, C141–147.
[19] Boveris, A., Martino, E. and Stoppani, A.O.M. (1977) Anal. Biochem. 80, 145–158.
[20] Nicholls, D.G. and Locke, R.M. (1984) Physiol. Rev. 64, 1–64.
[21] Halliwell, B. and Gutteridge, J.M.C. (1989) Free Radicals in Biology and Medicine, Oxford University Press, Oxford.