ANTIOXIDANTS & REDOX SIGNALING
Volume 4, Number 4, 2002

Original Research Communication

Membrane Depolarization of Isolated Rat Liver Mitochondria Attenuates Permeability Transition Pore Opening and Oxidant Production

ANNA ARONIS, RITA KOMARNITSKY, SHANI SHILO, AND OREN TIROSH

ABSTRACT

It has been suggested that one key feature of mitochondrial permeability transition (PT) regulation is its control by the proton electrochemical gradient and that depolarization favors pore opening, swelling, and reactive oxygen species (ROS) production. Moreover, ROS have been suggested to facilitate the process of mitochondrial PT pore opening. The aim of this study was to show that collapsing the mitochondrial membrane potential with the mitochondrial uncoupler, carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone (FCCP), at concentrations of up to 10 μ*M*, does not induce mitochondrial swelling and, in fact, stabilizes mitochondria exposed to oxidant, protecting them from *tert*-butyl hydroperoxide (TBH)-induced high-amplitude swelling. FCCP decreased polyethylene glycol-induced mitochondrial contraction following exposure to TBH, indicating closing of the PT mega-channel. In the presence of the calcium uniporter inhibitor ruthenium red, FCCP induced PT due to suppression of calcium efflux. Under PT-favorable conditions, ROS production was evaluated in mitochondria following treatments with TBH, inorganic phosphate, or FCCP (with or without ruthenium red). FCCP alone and in combination with ruthenium red attenuated mitochondria-derived ROS production. FCCP also decreased the augmented ROS production induced by inorganic phosphate. It is concluded that mitochondrial depolarization protects and prevents high-amplitude swelling and PT-derived ROS production. *Antioxid. Redox Signal.* 4, 647–654.

INTRODUCTION

In addition to their critical function in energy metabolism, mitochondria are known to regulate cell viability as well as cell death (4, 12, 20, 21, 28). Four interrelated mitochondrial pathways have been suggested to facilitate cell death: (a) mitochondrial permeability transition (MPT) and the release of cell death apoptotic-promotion factors; (b) cytochrome *c* release by proapoptotic members of the BCL-2 family of proteins (16); (c) disruption of ATP production, and (d) alteration of the cell's redox status and production of reactive oxygen species (ROS) (8, 11, 22, 23, 29, 32).

The permeability transition (PT) is an increase in the inner mitochondrial membrane's permeability most easily observed after matrix calcium accumulation (14). Although the PT can be favored by a large series of heterogeneous compounds and conditions, it is generally agreed that it is mediated by the opening of a cyclosporin A (CsA)-sensitive complex channel (31, 36).

Dissipation of the mitochondrial membrane potential is a hallmark of cell- death processes. This has been suggested to occur in both apoptosis and necrosis (21). It has been suggested that one key feature of MPT regulation is its control by the proton electrochemical gradient and that the pore is modulated by the transmembrane electrical potential difference (where depolarization favors pore opening) (2, 24, 31). Evidence that pore opening is voltage-controlled in intact isolated mitochondria is largely based on the effects of the

Institute of Biochemistry, Food Science and Nutrition, Faculty of Agricultural, Food and Environmental Quality Sciences, The Hebrew University of Jerusalem, Rehovot 76100, Israel.

protonophoric uncoupler carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone (FCCP), whereas other uncouplers have not been reported to induce PT (3).

Despite the evidence in the literature of MPT-promoting properties of FCCP, some articles have suggested that mitochondrial uncoupling by FCCP prevents cellular apoptosis (33). FCCP did not induce cytochrome *c* release and cell death in MH1C1 rat hepatoma cells, whereas arachidonic acid, another PT inducer, did (25). Recently, it has been reported that in mitochondria isolated from livers of healthy fasted rats, opening of the pore by active permeability agents such as inorganic phosphate (P_i) and the oxidant *tert*-butyl hydroperoxide (TBH) is associated with cytochrome *c* release into the supernatant of the suspensions; such release was not induced by exposure to an uncoupler (15).

ROS (TBH, peroxynitrite) have been suggested to facilitate the process of MPT pore opening (9, 19, 35). In addition, elevation in the production of endogenous mitochondrial ROS was demonstrated in P_i-induced PT (17). However, it is still not clear whether ROS are the effectors of PT or are simply the released product of several PT inducers. In a recent publication, induction of MPT was shown not to be facilitated by ROS as demonstrated by several antioxidants, which were unable to prevent PT (27).

One theory suggests that during uncoupling, the electron transport chain works more efficiently. The increase in efficiency leads to less leakage of electrons and therefore much lower ROS generation (5–7), obscuring the mechanism by which FCCP or lower membrane potential promotes pore opening.

It has therefore become critical to: (a) to evaluate the role of membrane potential during PT pore opening and (b) the role of ROS production in the induction of PT pore opening; (c) explore further the mechanism of elevated endogenous mitochondrial ROS production during the progress of P_i-induced PT; and (d) to search for common denominators explaining the PT effect of all inducers.

MATERIALS AND METHODS

Materials

Materials were obtained from the following sources: calcium chloride, rotenone, potassium hydrogen phosphate, FCCP, ruthenium red (RR), *p*-hydroxyphenylacetic acid (*p*-HPAA), and horseradish peroxidase (HRP) (Sigma, Rehovot, Israel); mannitol, sucrose, HEPES, EDTA, succinate, and TBH (Aldrich Chemical Co., Rehovot, Israel); polyethylene glycol 5000 monomethyl ether (PEG) (Fluka Chemie AG, Rehovot, Israel); dichlorodihydrofluorescein (H_2DCF), and MitoTracker Orange CMTMRos (Molecular Probes, Eugene, OR, U.S.A.).

Animals

Male Sprague–Dawley rats (4 months old and ~250 g) received food and water *ad libitum*. They were kept in plastic cages with wire tops in a room with controlled lighting. All animal experiments and handling protocols were approved by the ethics committee of the Faculty of Agricultural, Food and Environmental Quality Sciences, The Hebrew University of Jerusalem.

Isolation of rat liver mitochondria (RLM)

After an overnight fast, animals were anesthetized and killed by decapitation. Livers were removed with scissors and immediately immersed in ice-cold 210 m*M* mannitol, 70 m*M* sucrose, 5 m*M* HEPES, pH 7.35 (MSH buffer) containing 1 m*M* edta. Livers were freed of fat and connective tissue, cut to pieces with scissors, and homogenized in 60 ml of MSH/EDTA buffer per liver using a glass homogenizer with a Teflon pestle. Mitochondria were then isolated in MSH buffer by a conventional differential centrifugation method (30).

Standard incubation procedure

Isolated mitochondria (0.5–1 mg of protein/ml) were incubated at ambient temperature with calcium (60 μmol/mg of mitochondrial protein) with stirring, in a buffer consisting of 210 m*M* mannitol, 70 m*M* sucrose, 5 m*M* HEPES, pH 7.4. 5 m*M* succinate was used as a substrate in the presence of 2 μ*M* rotenone. Rotenone was added to succinate-energized mitochondria to prevent pyridine nucleotide oxidation by the electron-transfer chain during incubation and the PT treatments. Following the incubation, different treatments were applied as indicated in the figure legends.

ROS measurements

Intramitochondrial ROS were detected using H_2DCF (34). H_2DCF was selected for mitochondrial ROS measurements because (a) the probe is lipophilic, can cross membranes, and can be loaded and activated (deacetylated) inside the mitochondria, and (b) the probe is not positively charged and its accumulation by mitochondria is not dependent on the membrane potential. Mitochondria were resuspended and incubated with H_2DCF (25 μ*M*) for 10 min at room temperature. For the detection of ROS at the single mitochondrion level, fluorochrome-loaded mitochondria were excited using a 488-nm argon-ion laser in a flow cytometer (FACSort, BD). The dichlorofluorescein (DCF) emission was recorded at 530 nm. Data were collected from at least 50,000 mitochondria.

Production of peroxides was also evaluated by following total DCF fluorescence in the mitochondrial suspension using a microfluorometer plate reader (GENios, Tecan, Austria).

Determination of mitochondrial hydrogen peroxide (H_2O_2) release (extramitochondrial H_2O_2-like activity)

The release of H_2O_2 from isolated mitochondria was obtained using *p*-HPAA, which, when used as a substrate, is converted by HRP to a fluorescent compound in the presence of H_2O_2: 0.1 m*M* *p*-HPAA and 10 U/ml HRP at room temperature were used (13, 33).

Determination of mitochondrial membrane potential

Mitochondrial membrane potential was detected by flow cytometry (FACSort). For this assay, the membrane potential-

sensitive fluorescent probe MitoTracker Orange CMTMRos was used, at the following fluorescence settings: excitation at 488 nm and emission at 575 nm (FL2 channel). Isolated mitochondria (1 mg) were stained with 0.5 μM CMTMRos, samples were exposed to various treatments as described in the figure legends and analyzed for membrane potential. Under the experimental conditions used in this article, MitoTracker Orange CMTMRos did not induce any significant mitochondrial high-amplitude swelling (data not shown). The probe was found to be highly sensitive to changes in the energy status of the mitochondria.

Swelling and treatment with PEG_{5000}

Mitochondrial swelling was measured by the drop in A_{540} using a Spectronic Unicam spectrophotometer. Mitochondria were incubated in the standard incubation medium at 0.5 mg of mitochondrial protein/ml. PEG_{5000} (38 mM, 300 mOsmol) dissolved in water was added to mitochondrial solution at a ratio of 2 to 3 (26) in order to induce mitochondrial contraction. Mitochondrial contraction was immediately followed at OD_{540}.

Calcium measurements

Calcium efflux and influx were monitored with an Orion Ca^{2+} ion selective electrode (Orion Research Inc., Beverly, MA, U.S.A.). Mitochondria were incubated in the standard incubation medium with constant stirring at 1 mg of protein/ml.

Respiration control

Isolated mitochondria (0.5 mg of protein/ml) were incubated at ambient temperature. Mitochondrial oxygen consumption was measured polarographically using a computerized Clark-type oxygen electrode.

Statistics

The results shown are representative of a series of at least three experiments. Comparisons between multiple groups were made by analysis of variance; $p < 0.05$ was considered statistically significant.

RESULTS

FCCP-induced MPT in the presence and absence of RR

Collapsing the RLM membrane potential with the mitochondrial uncoupler, FCCP, at concentrations of up to 10 μM did not induce mitochondrial swelling (Fig. 1A). In fact, the treatment stabilized the mitochondria against high-amplitude swelling. Only when adding the calcium uniporter inhibitor RR at concentrations of 2 μM did the mitochondria become resensitized to FCCP-induced swelling: FCCP at concentrations as low as 0.2 μM induced rapid swelling, and at 10 μM FCCP, the combined effect with RR was further potentiated. RR alone did not induce high-amplitude swelling (Fig. 1A). CsA (1 μM) potently inhibited the observed swelling (Fig. 1B),

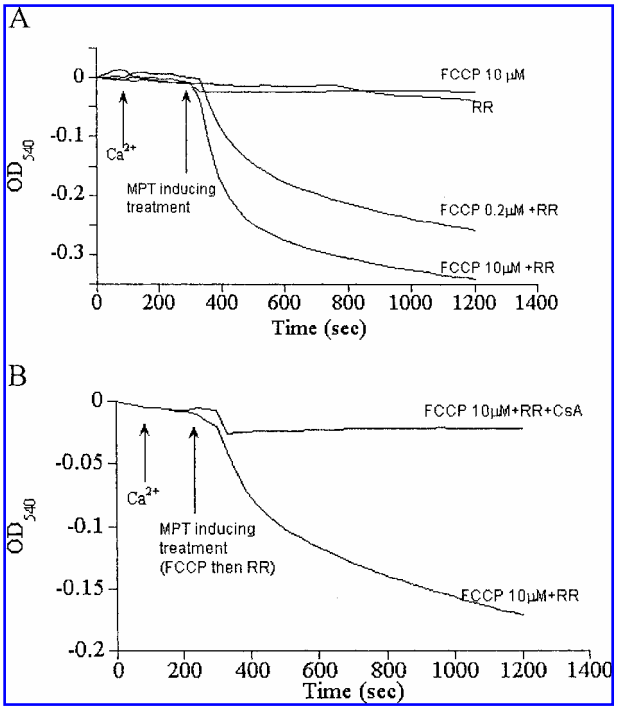

FIG. 1. Swelling induced by FCCP in the presence or absence of RR. Swelling was monitored at OD_{540}: **(A)** RR (2 μM) was added 20 s before the FCCP as MPT-inducing treatment. **(B)** CsA (1 μM) prevents PT pore opening by FCCP (10 μM) in the presence of RR. CsA was added to the mitochondrial incubation medium.

confirming that the combined treatment of FCCP and RR facilitated PT via opening of the mega-channel.

The mechanism by which RR promotes FCCP-dependent swelling is unclear. It has been hypothesized that inhibition of calcium efflux may contribute to the observed swelling. Measurement of calcium efflux from mitochondria revealed that RR significantly slows calcium release in mitochondria treated with FCCP (uncoupled mitochondria). Mitochondrial calcium retention in the presence of RR under depolarization conditions was observed in the presence of both low and high concentrations of FCCP (Fig. 2).

Protection by FCCP against oxidative stress

RLM loaded with calcium were exposed to oxidative stress. TBH (250 μM) promoted extensive swelling (Fig. 3A). FCCP added to the RLM after the exposure to oxidative stress prevented mitochondrial swelling in a dose-dependent fashion. At a concentration of 0.2 μM, FCCP attenuated the rate of occurrence and magnitude of the swelling, and at 10 μM, FCCP completely prevented TBH-induced swelling (Fig. 3A). In agreement with previous reports, CsA potently prevented TBH-induced swelling, confirming PT pore opening by the oxidant (data not shown). Following swelling, PEG was added to the mitochondrial solution. PEG induced shrinkage of the mitochondria due to removal of water through the open pore (Fig. 3B). Addition of FCCP, 2 min and 12 min after

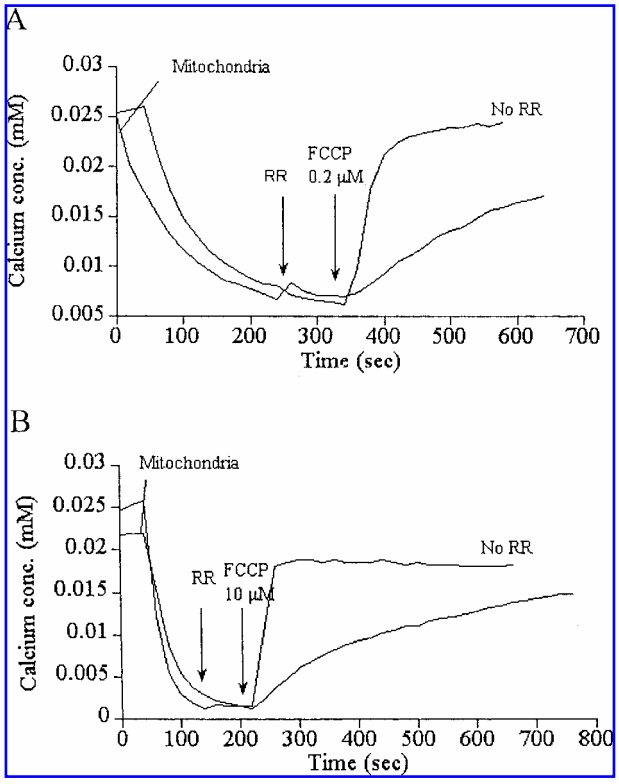

FIG. 2. Effect of RR and FCCP on calcium efflux. (A) Effect of low concentration of FCCP (0.2 μ*M*): the experiments were performed using 2 μ*M* RR followed by 0.2 μ*M* FCCP. **(B)** Effect of high concentration of FCCP (10 μ*M*).

TBH, attenuated the PEG-induced mitochondrial contraction, indicating that FCCP facilitated closing of the PT pore (Fig. 3B).

Measurements of calcium-retention capacity showed that FCCP potently releases calcium from the mitochondria, whereas in the absence of an uncoupler, calcium was retained in the matrix during swelling (up to 350 s after TBH); at 550 s after TBH, most calcium had left the matrix and swelling was significantly attenuated (Figs. 3A and 4A).

Calcium efflux was found to be redox-sensitive. A synergistic effect was observed when a low concentration of 0.2 μ*M* FCCP was used in combination with TBH. Treatment of mitochondria with TBH for 1 min prior to the addition of FCCP resulted in an almost twofold increase in the amount of calcium released from the mitochondria (Fig. 4B).

Evaluation of mitochondrial ROS generation during MPT

Oxidant treatment in mitochondria has been suggested to instigate the opening of the mega-channel. Total fluorescence was used to monitor ROS production. An analysis of the effects of FCCP in the presence or absence of RR indicated inhibition of endogenous mitochondrial ROS production during the MPT (Fig. 5A and B). Treatment with an uncoupler slowed ROS production relative to the untreated control (Fig. 5A and B). In addition to the effect of FCCP on calcium-loaded mitochondria undergoing PT (state 4 respiration), FCCP attenuated ROS production in mitochondria in state 3 respiration (data not shown). Using P_i, another PT-inducing treatment, elevation in endogenous mitochondrial ROS production was observed. In the presence of FCCP, the augmented ROS production in P_i-treated mitochondria was completely abrogated (Fig. 5C).

In addition to total fluorescence measurements, the oxidation status in the mitochondrial matrix was evaluated directly, using a flow cytometer. The isolated RLM showed rapid kinetics of DCF (the oxidized form of H_2DCF) accumulation in the matrix (data not shown). The different MPT-inducing treatments were then compared. The results of the total fluorescence reading (spectrofluorometer) reflected the oxidation status in the RLM matrix. P_i induced stronger matrix oxidation, whereas FCCP + RR suppressed the production of ROS (Fig. 6). TBH was used as a positive control and, indeed, such treatment elevated ROS in the RLM matrix (Fig. 6).

Alteration in membrane potential and mitochondrial respiration

All MPT-inducing treatments depolarized the mitochondria relative to untreated control. However, a high residual

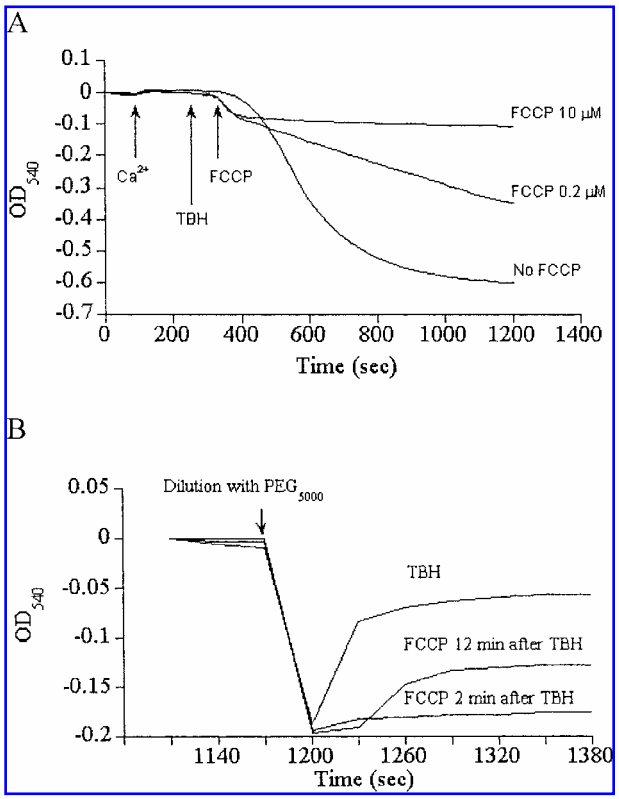

FIG. 3. Protective effect of FCCP against oxidants. (A) Swelling effect: FCCP was added after exposure to 250 μ*M* TBH. **(B)** PEG_{5000} induced contraction of mitochondria treated with 250 μ*M* TBH in the presence or absence of FCCP. TBH was added as in A, and FCCP was added where indicated 2 min and 12 min after the TBH.

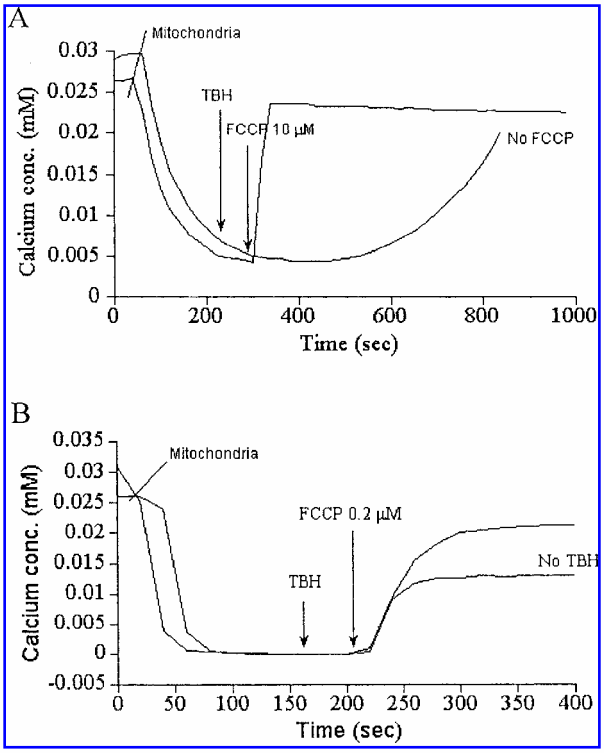

FIG. 4. Effect of TBH and FCCP on calcium efflux. **(A)** The experiments were performed using MPT-inducing treatment of 250 μ*M* TBH in the presence or absence of FCCP (10 μ*M*) that was added after the TBH. **(B)** Calcium efflux: synergistic effect of oxidant (250 μ*M* TBH) with FCCP.

membrane potential was observed in mitochondria treated with either TBH or P_i. These treatments resulted in partially polarized membranes, whereas FCCP (0.2 μ*M*) + RR further depolarized them (Fig. 7A). Control of mitochondria treated with FCCP (10 μ*M*) were used to demonstrate loss of membrane potential without PT.

As P_i was the one PT-inducing treatment that elevated endogenous mitochondrial ROS, mitochondrial oxygen consumption was monitored. An evaluation of oxygen consumption kinetics in mitochondrial respiration, in the absence of ADP, showed that both FCCP and P_i uncouple mitochondria and allow rapid oxygen consumption of all dissolved oxygen (Fig. 7B).

Effects of phosphate and TBH

Treatment with oxidant (TBH) consistently promoted less swelling than did P_i (Fig. 8A). The effect of P_i was faster and more potent than that of pure oxidant in RLM isolated from the same animal (Fig. 8A). According to extramitochondrial calcium measurements, TBH-induced swelling came to a halt following the calcium's release to the incubation medium. In contrast, P_i high-amplitude swelling was not accompanied by extensive mitochondrial calcium release (Fig. 8B). High concentrations of FCCP were unable to prevent P_i-induced swelling although FCCP totally prevented the effect of Pi augmenting ROS production (Fig. 5C.). However, FCCP attenuated the swelling amplitude (Fig 8C). FCCP did not induce calcium efflux from P_i treated mitochondria (data not shown).

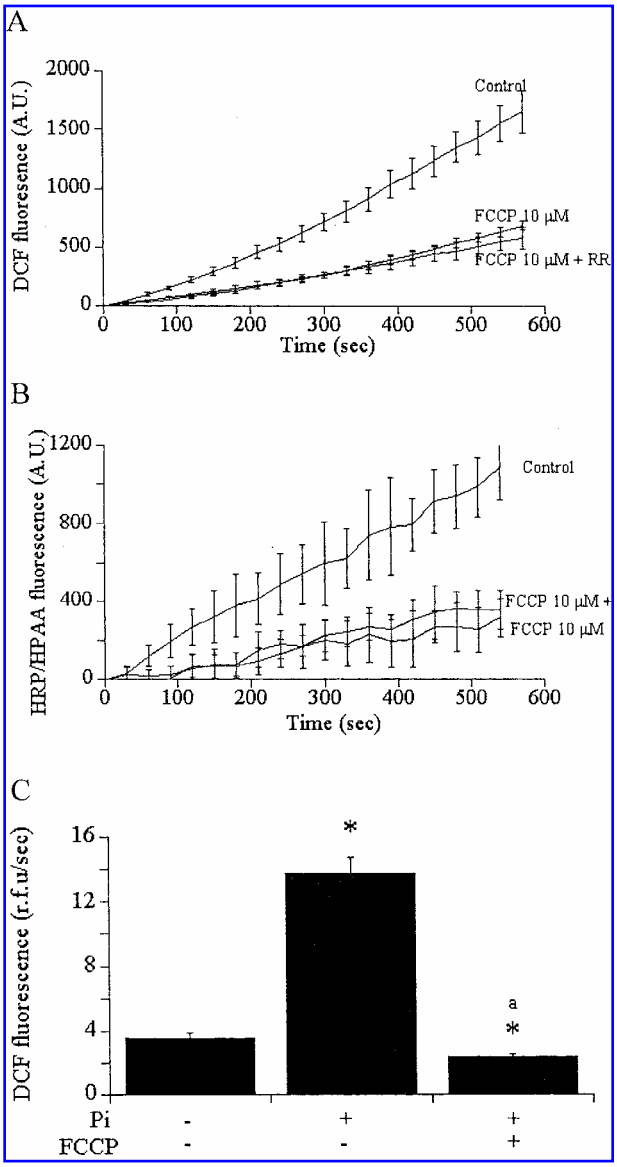

FIG. 5. Mitochondrial ROS (peroxide) production: regulation by FCCP, RR and P_i. **(A)** ROS accumulation kinetics: H_2DCF (25 μ*M*) was used as an oxidation-sensitive probe. FCCP (10 μ*M*) and RR (2 μ*M*) were added as MPT-inducing treatments, and the production of ROS was compared with controls. **(B)** ROS accumulation kinetics: HRP (10 U/ml) and *p*-HPAA (100 μ*M*) were used as oxidation-sensitive probes. FCCP (10 μ*M*) and RR (2 μ*M*) were added as MPT-inducing treatments, and the production of ROS was compared with controls. **(C)** Effect of P_i (20 m*M*): Mitochondria were loaded with H_2DCF. FCCP (10 μ*M*) prevented the augmented P_i-dependent ROS production. *Significantly different compared with control ($p < 0.05$). [a]Significantly different from P_i-treated mitochondria ($p < 0.05$).

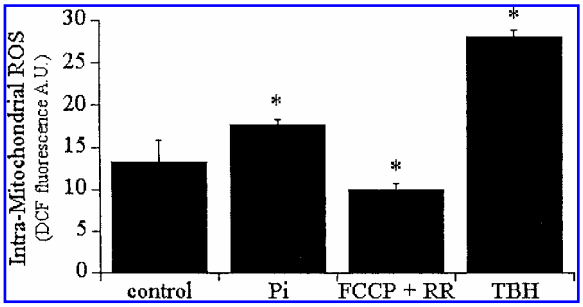

FIG. 6. Intramitochondrial ROS production measurements, effect of P_i, FCCP + RR, and TBH. P_i (20 m*M*), TBH (250 μ*M*), or FCCP (0.2 μ*M*) + RR (2 μ*M*) was added as MPT-inducing treatments, and the production of ROS was compared with controls. Mitochondria were then loaded with 25 μ*M* H_2DCF for 10 min and analyzed by flow cytometer. Fluorescence emission was monitored at the FL1 channel, and data were collected from 50,000 mitochondria. *Significantly different compared with controls ($p < 0.05$).

DISCUSSION

Collapsing mitochondrial membrane potential has been reported to promote calcium efflux from mitochondria, and this was suggested to facilitate mitochondrial damage (1). Here we show that mitochondria can protect themselves via this mechanism. In the presence of the calcium-uptake inhibitor RR, low membrane potential instigates PT pore opening. Slow calcium release in the presence of RR indicates that this calcium-uptake inhibitor can prevent such efflux under depolarization conditions, thereby facilitating PT.

Depolarization of the mitochondrial membrane prevented oxidant-induced swelling by closing the PT pore. FCCP could afford protection when added after exposure to oxidative stress. Therefore, attenuated membrane potential under oxidative stress may permit the mitochondria to regulate swelling by releasing calcium. Introducing a small amount of FCCP together with an oxidant exacerbated calcium release. This interesting observation implies that mitochondria exposed to oxidative stress are more receptive to low membrane potential-dependent calcium release.

Mitochondria are considered a major cellular source of ROS (6, 10). Increased mitochondrial ROS leakage was monitored in the presence of P_i (17, 18). Here we show that ROS production is potentiated by P_i. FCCP suppressed such ROS production, but did not prevent P_i-induced high-amplitude swelling. The kinetics of ROS production lagged behind the P_i-dependent PT pore opening. Therefore, ROS, although capable of instigating PT (TBH effect), cannot serve as the rate-limiting component in the process, but rather is a by-product of the P_i-induced PT. To understand the mechanism by which P_i treatment elevates mitochondrial ROS, oxygen consumption and membrane potential were measured. P_i had the same uncoupling effect as FCCP (accelerated oxygen consumption in the absence of ADP) as monitored by RLM oxygen consumption in the presence of high residual mitochondrial membrane potential. This demonstrates that higher membrane potential during PT pore opening in conjunction with accelerated electron flow favors the generation of ROS. Indeed, FCCP completely abrogated the ROS-production effect of P_i.

Conclusion

The data presented in this article demonstrate the following: (a) Low membrane potential attenuates high-amplitude swelling and prevents mitochondrial ROS production during PT pore opening. (b) High membrane potential during P_i-induced swelling and respiration uncoupling explain the observed increase in ROS production. (c) PT-induced swelling can be exacerbated independently of endogenous mitochondrial ROS production. (d) The presence of calcium in the ma-

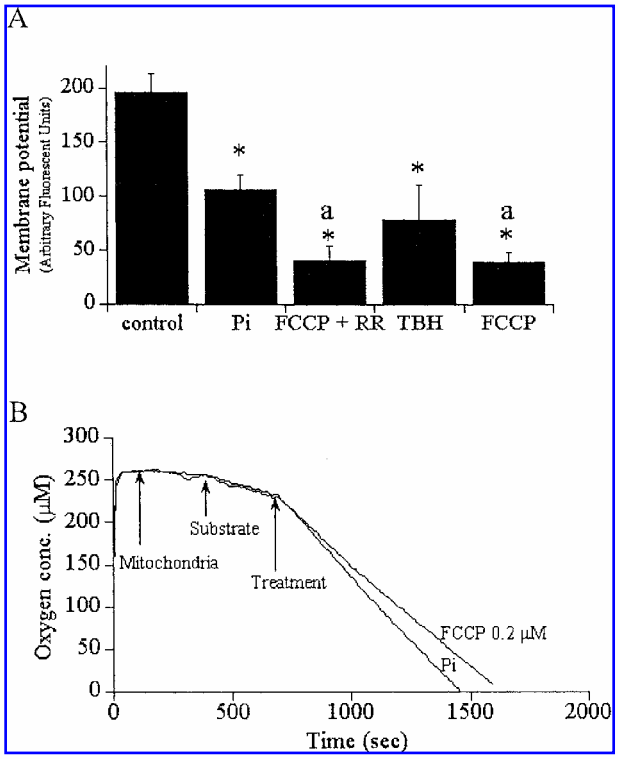

FIG. 7. Mitochondrial membrane potential and respiration control. (A) Membrane potential: mitochondria were incubated with 0.5 μ*M* of the membrane potential-sensitive probe CMTMRos, and membrane potential was evaluated 5 min after MPT treatment by flow cytometer. Fluorescence emission was recorded at FL2, and data were collected from 50,000 mitochondria. P_i (20 m*M*), FCCP (0.2 μ*M*) + RR (2 μ*M*), or TBH (250 μ*M*) was added as MPT-inducing treatments, and the membrane potential was compared with control. FCCP (10 μ*M*) was used as control for complete mitochondrial depolarization (right column). *Significantly different compared with control ($p < 0.05$). [a]Significantly different from P_i-treated mitochondria ($p < 0.05$). **(B)** Mitochondrial respiration: the incubation medium contained MSH buffer, pH 7.4, to a final volume of 2.5 ml. Mitochondria (0.5 mg of protein/ml) were used; 5 m*M* succinate, 2 μ*M* rotenone, and Ca^{2+} (60 nmol/mg of protein) were then added (substrate). Both 0.2 μ*M* FCCP and 20 m*M* P_i induced accelerated mitochondrial respiration (treatment).

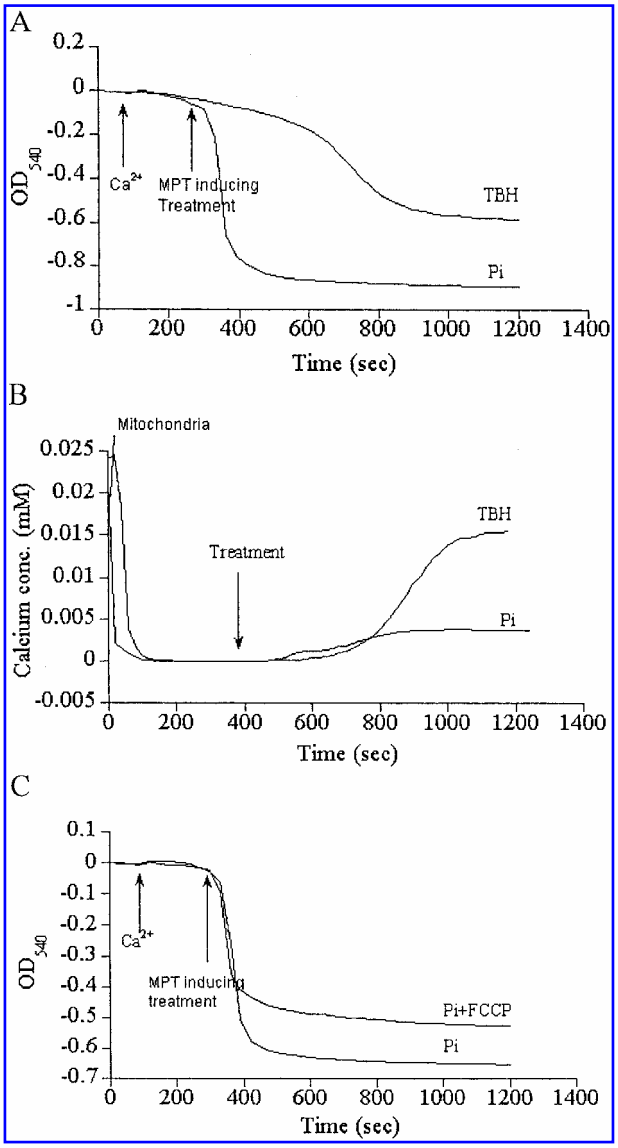

FIG. 8. Effect of TBH and Pi on PT pore opening. (A) Swelling effect monitored at OD_{540}: TBH (250 μM) or P_i (20 mM) was added as MPT-inducing treatments. **(B)** Calcium efflux: experiments were performed using the same MPT-inducing treatment as in A. **(C)** Swelling effect monitored at OD_{540}: 10 μM FCCP and 20 mM P_i were used (MPT-inducing treatment).

trix is an obligatory component for the progression of high-amplitude swelling, because with both P_i and RR (in the presence of FCCP), calcium is retained in the matrix during mitochondrial swelling and the effect of TBH is prevented by FCCP-induced calcium release.

Perspective for redox signaling

As mitochondria are one of the most important cellular sources of ROS production, during programmed cell death attenuation of membrane potential might lead to low ROS generation. For example, during receptor-mediated apotosis, inhibition of ROS production and low mitochondrial membrane potential were observed by us (unpublished observations). Such a redox imbalance (low ROS) may alter redox-signaling processes that are oxidant-dependent and vital to the cell facilitating process of cell death.

ACKNOWLEDGMENTS

This work was supported by internal grant 0346382 of the Hebrew University of Jerusalem.

ABBREVIATIONS

CsA, cyclosporin A; DCF, dichlorofluorescein; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; H_2DCF, dichlorodihydrofluorescein; H_2O_2, hydrogen peroxide; *p*-HPAA, hydroxyphenylacetic acid; HRP, horseradish peroxidase; MPT, mitochondrial permeability transition; P_i, inorganic phosphate; PEG, polyethylene glycol 5000 monomethyl ether; PT, permeability transition; RLM, rat liver mitochondria; ROS, reactive oxygen species; RR, ruthenium red; TBH, *tert*-butyl hydroperoxide.

REFERENCES

1. Beatrice MC, Palmer JW, and Pfeiffer DR. The relationship between mitochondrial membrane permeability, membrane potential, and the retention of Ca^{2+} by mitochondria. *J Biol Chem* 255: 8663–8671, 1980.
2. Bernardi P. Modulation of the mitochondrial cyclosporin A-sensitive permeability transition pore by the proton electrochemical gradient. Evidence that the pore can be opened by membrane depolarization. *J Biol Chem* 267: 8834–8839, 1992.
3. Bernardi P and Petronilli V. The permeability transition pore as a mitochondrial calcium release channel: a critical appraisal. *J Bioenerg Biomembr* 28: 131–138, 1996.
4. Bernardi P, Petronilli V, Di Lisa F, and Forte M. A mitochondrial perspective on cell death. *Trends Biochem Sci* 26: 112–117, 2001.
5. Boveris A and Chance B. The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. *Biochem J* 134: 707–716, 1973.
6. Boveris A, Oshino N, and Chance B. The cellular production of hydrogen peroxide. *Biochem J* 128: 617–630, 1972.
7. Budd SL, Castilho RF, and Nicholls DG. Mitochondrial membrane potential and hydroethidine-monitored superoxide generation in cultured cerebellar granule cells. *FEBS Lett* 415: 21–24, 1997.
8. Cai J and Jones DP. Mitochondrial redox signaling during apoptosis. *J Bioenerg Biomembr* 31: 327–334, 1999.
9. Costantini P, Chernyak BV, Petronilli V, and Bernardi P. Modulation of the mitochondrial permeability transition pore by pyridine nucleotides and dithiol oxidation at two separate sites. *J Biol Chem* 271: 6746–6751, 1996.

10. Dawson TL, Gores GJ, Nieminen AL, Herman B, and Lemasters JJ. Mitochondria as a source of reactive oxygen species during reductive stress in rat hepatocytes. *Am J Physiol* 264: C961–C967, 1993.
11. Diehl AM and Hoek JB. Mitochondrial uncoupling: role of uncoupling protein anion carriers and relationship to thermogenesis and weight control "the benefits of losing control." *J Bioenerg Biomembr* 31: 493–506, 1999.
12. Green DR and Reed JC. Mitochondria and apoptosis. *Science* 281: 1309–1312, 1998.
13. Guilbault GG, Brignac PJ Jr, and Juneau M. New substrates for the fluorometric determination of oxidative enzymes. *Anal Chem* 40: 1256–1263, 1968.
14. Gunter TE and Pfeiffer DR. Mechanisms by which mitochondria transport calcium. *Am J Physiol* 258: C755–C786, 1990.
15. Kantrow SP, Tatro LG, and Piantadosi CA. Oxidative stress and adenine nucleotide control of mitochondrial permeability transition. *Free Radic Biol Med* 28: 251–260, 2000.
16. Korsmeyer SJ, Wei MC, Saito M, Weiler S, Oh KJ, and Schlesinger PH. Pro-apoptotic cascade activates BID, which oligomerizes BAK or BAX into pores that result in the release of cytochrome *c*. *Cell Death Differ* 7: 1166–1173, 2000.
17. Kowaltowski AJ, Castilho RF, Grijalba MT, Bechara EJ, and Vercesi AE. Effect of inorganic phosphate concentration on the nature of inner mitochondrial membrane alterations mediated by Ca^{2+} ions. A proposed model for phosphate-stimulated lipid peroxidation. *J Biol Chem* 271: 2929–2934, 1996.
18. Kowaltowski AJ, Netto LE, and Vercesi AE. The thiol-specific antioxidant enzyme prevents mitochondrial permeability transition. Evidence for the participation of reactive oxygen species in this mechanism. *J Biol Chem* 273: 12766–12769, 1998.
19. Kowaltowski AJ, Castilho RF, and Vercesi AE. Mitochondrial permeability transition and oxidative stress. *FEBS Lett* 495: 12–15, 2001.
20. Kroemer G and Reed JC. Mitochondrial control of cell death. *Nat Med* 6: 513–519, 2000.
21. Kroemer G, Zamzami N, and Susin SA. Mitochondrial control of apoptosis. *Immunol Today* 18: 44–51, 1997.
22. Lee HC and Wei YH. Mitochondrial role in life and death of the cell. *J Biomed Sci* 7: 2–15, 2000.
23. Patel M and Day BJ. Metalloporphyrin class of therapeutic catalytic antioxidants. *Trends Pharmacol Sci* 20: 359–364, 1999.
24. Petronilli V, Nicolli A, Costantini P, Colonna R, and Bernardi P. Regulation of the permeability transition pore, a voltage-dependent mitochondrial channel inhibited by cyclosporin A. *Biochim Biophys Acta* 1187: 255–259, 1994.
25. Petronilli V, Penzo D, Scorrano L, Bernardi P, and Di Lisa F. The mitochondrial permeability transition, release of cytochrome *c* and cell death. Correlation with the duration of pore openings in situ. *J Biol Chem* 276: 12030–12034, 2001.
26. Pfeiffer DR, Gudz TI, Novgorodov SA, and Erdahl WL. The peptide mastoparan is a potent facilitator of the mitochondrial permeability transition. *J Biol Chem* 270: 4923–4932, 1995.
27. Qu B, Li Q, Wong KP, Tan TM, and Halliwell B. Mechanism of clofibrate hepatotoxicity: mitochondrial damage and oxidative stress in hepatocytes. *Free Radic Biol Med* 31: 659–669, 2001.
28. Reed JC. Double identity for proteins of the Bcl-2 family. *Nature* 387: 773–776, 1997.
29. Richter C, Gogvadze V, Laffranchi R, Schlapbach R, Schweizer M, Suter M, Walter P, and Yaffee M. Oxidants in mitochondria: from physiology to diseases. *Biochim Biophys Acta* 1271: 67–74, 1995.
30. Schweizer M and Richter C. Peroxynitrite stimulates the pyridine nucleotide-linked Ca^{2+} release from intact rat liver mitochondria. *Biochemistry* 35: 4524–4528, 1996.
31. Scorrano L, Petronilli V, and Bernardi P. On the voltage dependence of the mitochondrial permeability transition pore. A critical appraisal. *J Biol Chem* 272: 12295–12299, 1997.
32. Simon HU, Haj-Yehia A, and Levi-Schaffer F. Role of reactive oxygen species (ROS) in apoptosis induction. *Apoptosis* 5: 415–418, 2000.
33. Tan S, Sagara Y, Liu Y, Maher P, and Schubert D. The regulation of reactive oxygen species production during programmed cell death. *J Cell Biol* 141: 1423–1432, 1998.
34. Tirosh O, Sen CK, Roy S, and Packer L. Cellular and mitochondrial changes in glutamate-induced HT4 neuronal cell death. *Neuroscience* 97: 531–541, 2000.
35. Zago EB, Castilho RF, and Vercesi AE. The redox state of endogenous pyridine nucleotides can determine both the degree of mitochondrial oxidative stress and the solute selectivity of the permeability transition pore. *FEBS Lett* 478: 29–33, 2000.
36. Zoratti M and Szabo I. The mitochondrial permeability transition. *Biochim Biophys Acta* 1241: 139–176, 1995.

Address reprint requests to:
Dr. Oren Tirosh
Institute of Biochemistry, Food Science and Nutrition
The Hebrew University of Jerusalem
Rehovot 76100, Israel

E-mail: otirosh@agri.huji.ac.il

Received for publication January 19, 2002; accepted April 16, 2002.

This article has been cited by:

1. Siok-Koon Yeo, Min-Tze Liong. 2012. Enhanced growth and bioconversion of isoflavones in prebiotic-soymilk fermented by UV-treated lactobacilli and bifidobacteria. *International Journal of Food Sciences and Nutrition* **63**:5, 566-579. [CrossRef]
2. T. Zampolla, E. Spikings, D. Rawson, T. Zhang. 2011. Cytoskeleton proteins F-actin and tubulin distribution and interaction with mitochondria in the granulosa cells surrounding stage III zebrafish (Danio rerio) oocytes. *Theriogenology* . [CrossRef]
3. João P. Monteiro, André F. Martins, Marlene Lúcio, Salette Reis, Carlos F. G. C. Geraldes, Paulo J. Oliveira, Amália S. Jurado. 2011. Interaction of carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP) with lipid membrane systems: a biophysical approach with relevance to mitochondrial uncoupling. *Journal of Bioenergetics and Biomembranes* **43**:3, 287-298. [CrossRef]
4. Luciene M. Zanchetta, Amaya Garcia, Fiona Lyng, James Walsh, James E. J. Murphy. 2011. Mitophagy and mitochondrial morphology in human melanoma-derived cells post exposure to simulated sunlight. *International Journal of Radiation Biology* **87**:5, 506-517. [CrossRef]
5. Jae Myeong Lee, Jung Kook Suh, Ji Seon Jeong, Sang Yun Cho, Dong Won Kim. 2010. Antioxidant effect of lidocaine and procaine on reactive oxygen species-induced endothelial dysfunction in the rabbit abdominal aorta. *Korean Journal of Anesthesiology* **59**:2, 104. [CrossRef]
6. Seung Yoon Lee, Jung Kook Suh, Jin Hwa Choi, Woo Jae Jeon, Mi Ae Cheong. 2010. Effect of ketorolac and diclofenac on the impairment of endothelium-dependent relaxation induced by reactive oxygen species in rabbit abdominal aorta. *Korean Journal of Anesthesiology* **59**:3, 196. [CrossRef]
7. Miryam Calvino-Fernández, Selma Benito-Martínez, Trinidad Parra-Cid. 2008. Oxidative stress by Helicobacter pylori causes apoptosis through mitochondrial pathway in gastric epithelial cells. *Apoptosis* **13**:10, 1267-1280. [CrossRef]
8. Nathan R. Brady , Anne Hamacher-Brady , Hans V. Westerhoff , Roberta A. Gottlieb . 2006. A Wave of Reactive Oxygen Species (ROS)-Induced ROS Release in a Sea of Excitable Mitochondria. *Antioxidants & Redox Signaling* **8**:9-10, 1651-1665. [Abstract] [Full Text PDF] [Full Text PDF with Links]
9. Zita Bognar, Tamas Kalai, Anita Palfi, Katalin Hanto, Balazs Bognar, Laszlo Mark, Zoltan Szabo, Antal Tapodi, Balazs Radnai, Zsolt Sarszegi, Arpad Szanto, Ferenc Gallyas, Kalman Hideg, Balazs Sumegi, Gabor Varbiro. 2006. A novel SOD-mimetic permeability transition inhibitor agent protects ischemic heart by inhibiting both apoptotic and necrotic cell death. *Free Radical Biology and Medicine* **41**:5, 835-848. [CrossRef]
10. Shani Shilo , Michal Aharoni-Simon , Oren Tirosh . 2005. Selenium Attenuates Expression of MnSOD and Uncoupling Protein 2 in J774.2 Macrophages: Molecular Mechanism for Its Cell-Death and Antiinflammatory Activity. *Antioxidants & Redox Signaling* **7**:1-2, 276-286. [Abstract] [Full Text PDF] [Full Text PDF with Links]
11. Nathan R. Brady, Steven P. Elmore, Johannes J.H.G.M. van Beek, Klaas Krab, Pierre J. Courtoy, Louis Hue, Hans V. Westerhoff. 2004. Coordinated Behavior of Mitochondria in Both Space and Time: A Reactive Oxygen Species-Activated Wave of Mitochondrial Depolarization. *Biophysical Journal* **87**:3, 2022-2034. [CrossRef]
12. Gustav Mattiasson. 2004. Flow cytometric analysis of isolated liver mitochondria to detect changes relevant to cell death. *Cytometry* **60A**:2, 145-154. [CrossRef]
13. Cristina Bianchi, Romana Fato, Alessia Angelin, Fabiana Trombetti, Vittoria Ventrella, Anna Rosa Borgatti, Ernesto Fattorusso, Patrizia Ciminiello, Paolo Bernardi, Giorgio Lenaz, Giovanna Parenti Castelli. 2004. Yessotoxin, a shellfish biotoxin, is a potent inducer of the permeability transition in isolated mitochondria and intact cells. *Biochimica et Biophysica Acta (BBA) - Bioenergetics* **1656**:2-3, 139-147. [CrossRef]

14. 2003. Trend of Most Cited Papers (2001-2002) in ARS. *Antioxidants & Redox Signaling* **5**:6, 813-815. [Citation] [Full Text PDF] [Full Text PDF with Links]

15. Shani Shilo , Oren Tirosh . 2003. Selenite Activates Caspase-Independent Necrotic Cell Death in Jurkat T Cells and J774.2 Macrophages by Affecting Mitochondrial Oxidant Generation. *Antioxidants & Redox Signaling* **5**:3, 273-279. [Abstract] [Full Text PDF] [Full Text PDF with Links]