Free Radical Research, Volume 38 Number 10 (October 2004), pp. 1113–1118

Taylor & Francis
healthsciences

Phosphate Increases Mitochondrial Reactive Oxygen Species Release

GRACIELE A. OLIVEIRA and ALICIA J. KOWALTOWSKI*

Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, SP, Brazil

Accepted by Professor H. Sies

(Received 20 June 2004; In revised form 12 August 2004)

The effects of inorganic phosphate (P_i), the main intracellular membrane permeable anion capable of altering mitochondrial pH gradients (ΔpH), were measured on mitochondrial H_2O_2 release. As expected, P_i decreased ΔpH and increased the electric membrane potential (ΔΨ). Mitochondrial H_2O_2 release was stimulated by P_i and also by its structural analogue arsenate. However, acetate, another membrane-permeable anion, did not stimulate mitochondrial H_2O_2 release. The stimulatory effect promoted by P_i was prevented by CCCP, which decreases transport of P_i across the inner mitochondrial membrane, indicating that P_i must be in the mitochondrial matrix to stimulate H_2O_2 release. In conclusion, we found that P_i and arsenate stimulate mitochondrial reactive oxygen release, an effect that may contribute towards oxidative stress under conditions such as ischemia/reperfusion, in which high-energy phosphate bonds are hydrolyzed.

Keywords: Brain; Mitochondria; Membrane potential; Hydrogen peroxide; Free radical; Anion transport

Abbreviations: ΔpH, difference in pH across the inner mitochondrial membrane; Δp, protonmotive force; ΔΨ, mitochondrial inner membrane potential; A.U., arbitrary units; BCECF, 2′, 7′-bis (2-carboxyethyl)-5(6)-carboxyfluorescein; BCECF-AM, 2′, 7′-bis (2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester; BSA, bovine serum albumin; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; EGTA, ethylene glycol-bis(2-aminoethylether)-N, N, N′, N′-tetraacetic acid; HRP, horseradish peroxidase; P_i, inorganic phosphate; RBM, rat brain mitochondria

INTRODUCTION

The mitochondrial electron transport chain continuously produces water from O_2 through four consecutive one-electron reductions at the level of cytochrome c oxidase. The electrons reach cytochrome c oxidase by sequential transfers from other transport chain components, and are initially removed from NADH and $FADH_2$. During these transfers, a small portion of the electrons is sidetracked to reduce O_2 at intermediate steps, mainly within Complexes I and III. This monoelectronic reduction of O_2 results in the formation of the superoxide radical anion, which can be dismutated to H_2O_2 and transformed into other reactive oxygen species (ROS—for reviews, see Refs. [1–4]). As a result, mitochondria are a major intracellular source of ROS, which can affect cell function and integrity. As an example, small increases in mitochondrial ROS act as signals to activate protective mechanisms such as ischemic preconditioning,[5,6] preventing subsequent oxidative stress.[6–8] More intense changes in mitochondrial ROS release have been implicated in tissue damage occurring in a variety of conditions including ischemia/reperfusion, Parkinson's disease and aging.[1–4,9,10] It is thus of utmost importance to have a good understanding of physiological and pathological phenomena which can affect mitochondrial ROS generation

Mitochondrial protonmotive force (Δp) is the regulator of electron transport rates, and, presumably, should affect electron leakage and ROS generation. Δp has two components: (i) the inner membrane potential (ΔΨ), or the difference in electrical potential across the inner membrane, and (ii) ΔpH, the difference in proton concentrations across the inner membrane.[11] In mitochondria, ΔpH is the smaller

*Corresponding author. Address: Av. Prof. Lineu Prestes, 748, 05508-900, São Paulo, SP, Brazil. Fax: + 55-11-38155579.
E-mail: alicia@iq.usp.br

ISSN 1071-5762 print/ISSN 1029-2470 online © 2004 Taylor & Francis Ltd
DOI: 10.1080/10715760400009258

component of Δp due to the abundance of membrane-permeable weak acids which act as buffers.[11] Since electron transport rates are controlled by Δp, tending toward a steady-state, a lack of membrane-permeable weak acids is expected to increase ΔpH and decrease $\Delta\Psi$.[11] The equation $\Delta p = \Delta\Psi - (2.3RT \cdot F^{-1})\Delta pH$, in which R is the gas constant, F is the Faraday constant and T is the absolute temperature, correlates values of Δp, $\Delta\Psi$ and ΔpH.

The relationship between $\Delta\Psi$ and ROS release is well established. Decreases in $\Delta\Psi$ such as those promoted by oxidative phosphorylation,[12] fatty acid transport,[13] uncoupling proteins[14,15] or K^+ cycling[8,16] prevent mitochondrial ROS formation. Indeed, the prevention of mitochondrial ROS release is believed to be one of the main functions of uncoupling proteins, which are strongly activated by mitochondrial superoxide radicals.[17] This prevention is related to enhanced rates of electron transfer associated with larger membrane permeability to protons, limiting the lifetime of intermediates capable of monoelectronic O_2 reduction. Increased electron transport rates may also decrease local oxygen tensions, further preventing ROS formation.[18] Although these effects have always been related to $\Delta\Psi$ due to its ease of measurement and predominant role in Δp, the experiments were conducted in the presence of constant concentrations of membrane-permeable weak acids, and $\Delta\Psi$ was regulated by increasing inner membrane permeability to protons, an effect which would also alter Δp.

Inorganic phosphate (P_i) is the main intracellular anion capable of entering the mitochondrial matrix through a P_i/OH^- exchanger[19] and buffering intramitochondrial pH. P_i transport into mitochondria is stimulated by ΔpH and cation transport, such as K^+ entry through ATP-sensitive K^+ channels.[20] In addition, intracellular P_i concentrations can increase under conditions in which energy metabolism is compromised due to high-energy phosphate bond hydrolysis. Thus, mitochondrial P_i uptake can vary widely under conditions in which mitochondrial ROS release is altered and may affect cell viability. Despite this knowledge, little is known about the direct effects of P_i uptake on mitochondrial ROS release. Here, we investigated the results of P_i transport on mitochondrial Δp, $\Delta\Psi$, ΔpH and H_2O_2 generation, as a measure of mitochondrial ROS production. We also compared the effects of P_i to that of other membrane-permeable anions.

MATERIALS AND METHODS

Mitochondrial Isolation

Rat brain, heart and liver mitochondria were isolated as described previously.[8,21,22] All experiments were conducted within 3 h of mitochondrial isolation. Protein concentrations were determined using the Biuret method.

H_2O_2 Release Measurements

H_2O_2 release from mitochondria was measured using Amplex® Red (Molecular Probes, Eugene, OR, USA) which is oxidized in the presence of extramitochondrial horseradish peroxidase (HRP) bound to H_2O_2 generating resorufin with a 1:1 stoichiometry.[23] This reaction is unaltered by added P_i, arsenate or acetate, as determined by calibrations using known quantities of H_2O_2. Resorufin fluorescence was monitored using a temperature controlled (37°C) Hitachi 4500 fluorescence spectrophotometer operating at excitation and emission wavelengths of, respectively, 563 and 587 nm, with 5 nm slits and continuous stirring. H_2O_2 release rates (Figs. 2–4) were calculated from the average increase in fluorescence between 0.5 and 2 min after the addition of P_i, arsenate or acetate.

Mitochondrial Membrane Potential ($\Delta\Psi$)

Mitochondrial $\Delta\Psi$ was estimated through fluorescence changes of safranine O (5 μM) at 37°C with excitation and emission wavelengths of, respectively, 495 and 586 nm, with 5 nm slits. Data were calibrated using a K^+ gradient as described byÅkerman and Wikstrom[24] and Kowaltowski *et al.*[25] Briefly, mitochondria are incubated in K^+-free buffer in the presence of 100 nM valinomycin, to allow K^+ distribution across the membrane, and fluorescence is measured as increasing extramitochondrial K^+ additions are made. $\Delta\Psi$ is calculated for each K^+ concentration using the Nernst equation, $\Delta\Psi = 60 * \log([K^+_{in}]/[K^+_{out}])$, where K^+_{in} is the intramitochondrial K^+ concentration (assumed to be 150 mM) and K^+_{out} is the added K^+ concentration. Calculated $\Delta\Psi$ values are plotted against measured fluorescence values, and the best fitting for the resulting plot is used to transform other fluorescence traces into $\Delta\Psi$ measurements (see Ref. [25] for an experimental tracing of a $\Delta\Psi$ calibration using this method).

Intramitochondrial PH

Mitochondria were suspended in medium containing 2 μM BCECF-AM (Molecular Probes), 75 mM sucrose, 225 mM manitol, 5 mM Hepes, 1 mM EGTA and 1 mg/ml bovine serum albumin, pH 7.2 (KOH) and incubated at 4°C for 20 min. The BCECF-loaded mitochondria were then washed in buffer devoid of BCECF, centrifuged, and ressuspended in the same medium. BCECF fluorescence emission was measured at 535 nm with variable excitation wavelengths, at 37°C. Intramitochondrial pH was calculated from

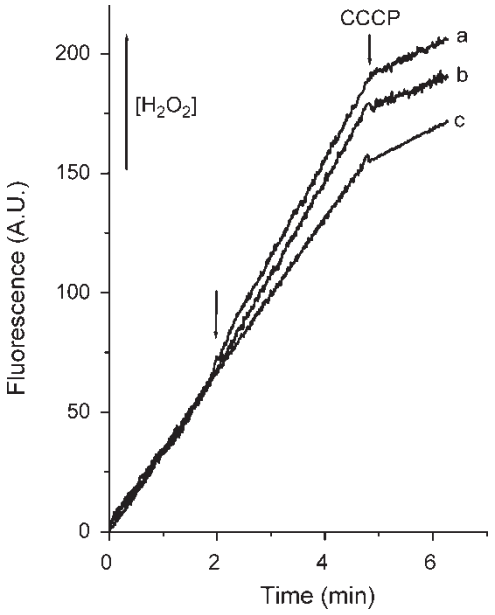

FIGURE 1 P_i and arsenate increase H_2O_2 release. Rat brain mitochondria (RBM, 0.5 mg/ml) were added to reaction media at 37°C containing 150 mM KCl, 10 mM Hepes, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM malate, 2 mM glutamate, 1 mg/ml BSA and 1 μg/ml oligomycin, pH 7.2 (KOH). Amplex® Red (50 μM) and 1 U/ml HRP were present to measure H_2O_2 release (see "Materials and Methods" Section 2) under control conditions (no further additions, line c) or in the presence of 2 mM P_i (line a) or arsenate (line b) added where indicated by the arrow. CCCP (2 μM) was added to all traces where indicated. The results shown are representative of > 5 similar repetitions.

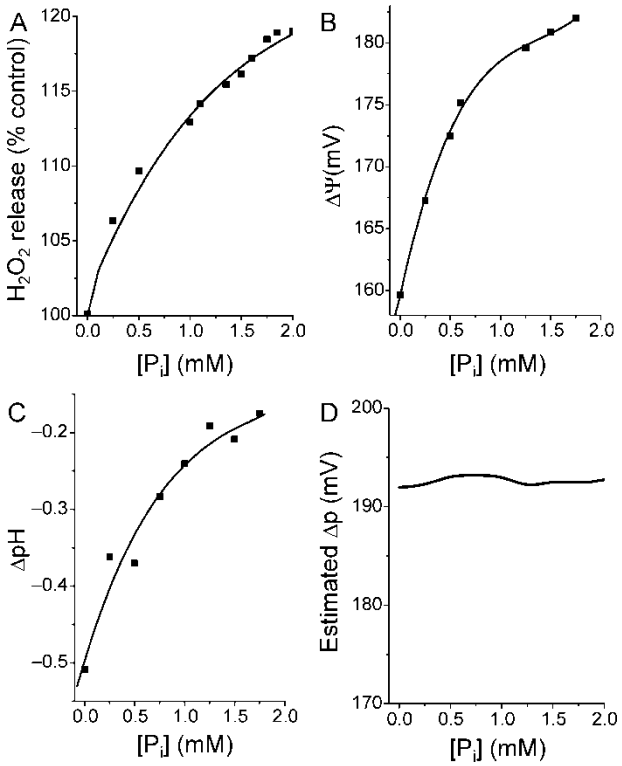

FIGURE 2 P_i effect on mitochondrial H_2O_2 release, ΔΨ, ΔpH and Δp. RBM (0.5 mg/ml) were added to the reaction media described in Fig. 1 in the presence of 50 μM Amplex® Red and 1 U/ml HRP (Panel A) or 5 μM safranine O (Panel B) to measure H_2O_2 release and ΔΨ, respectively (see "Materials and Methods"). Mitochondria in Panel C were pre-loaded with BCECF to measure matrix pH as described in "Materials and Methods". P_i was added at the concentrations indicated. The results shown were conducted in parallel and are representative of at least 3 similar repetitions. Panel D represents estimated Δp values calculated by applying the formula $\Delta p = \Delta\Psi - 61\Delta pH$ to the fittings for data in Panels B and C.

the ratio between fluorescence levels at 490 and 440 nm as described by the supplier (see Ref. [25]). All experiments were conducted within 30 min of mitochondrial loading with BCECF.

RESULTS

Using the highly sensitive H_2O_2 detection system consisting of Amplex Red in the presence of HRP, H_2O_2 release from brain mitochondria respiring on malate plus glutamate in the absence of oxidative phosphorylation (state 4) and added P_i is detectable (Fig. 1, line c). As expected, H_2O_2 release under these conditions is strongly decreased when respiration is stimulated by the addition of the proton ionophore CCCP.[12] Interestingly, the addition of P_i to the reaction media leads to a significant and constant increment in Amplex® Red oxidation rates (line a). This increase is not related to enhanced Amplex® Red sensitivity to H_2O_2 as suggested by the finding that rates in the presence of CCCP were unaltered by P_i (line a) and that P_i does not change the sensitivity of this probe to added H_2O_2 (see "Materials and Methods"). Furthermore, P_i increased H_2O_2 release in liver (up to 22%) and heart mitochondria (up to 28%), indicating that P_i can regulate mitochondrial ROS release in a variety of tissues. Arsenate, a structural analogue of P_i, also increased mitochondrial H_2O_2 release (line b), to a slightly lower extent.

The P_i concentrations which promoted increases in brain mitochondrial H_2O_2 release (Fig. 2, Panel A) were similar to those that lead to increments in ΔΨ (Panel B) and decreases in ΔpH (Panel C). Δp, estimated using the polynomial fittings to data in Panels B and C and applying the formula $\Delta p = \Delta\Psi - 61\Delta pH$ (see "Introduction") remained practically unaltered under these conditions (Panel D). These experiments suggest the P_i effect on H_2O_2 release is not related to changes in Δp, and may be caused by variations in ΔΨ and/or ΔpH.

Next, we tested the effects of two other membrane-permeable anions capable of buffering mitochondrial pH (Fig. 3): the P_i analogue arsenate (■) and acetate (▲), which is structurally unrelated to P_i. Surprisingly, acetate, at concentrations which increased ΔΨ (Panel A), lead to decreased brain mitochondrial H_2O_2 release (Panel B). This effect was not due to the sensitivity of the H_2O_2 detection system in the presence of added acetate (see "Materials and Methods"). Arsenate increased H_2O_2 release

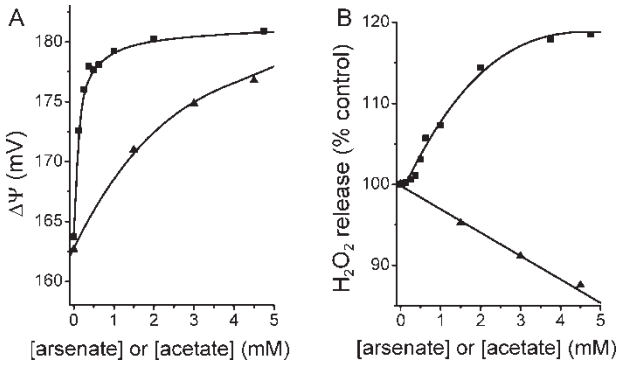

FIGURE 3 Effects of arsenate and acetate on mitochondrial H_2O_2 release and $\Delta\Psi$. RBM were incubated under the conditions described in Fig. 2. $\Delta\Psi$ (Panel A) and H_2O_2 release (Panel B) were measured in the presence of the concentrations of arsenate (■) or acetate (▲) indicated. The results shown are representative of 3 similar repetitions.

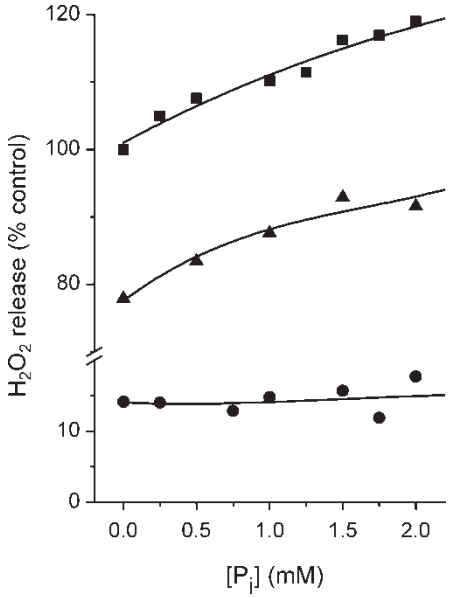

FIGURE 4 CCCP prevents P_i-increased H_2O_2 release. RBM were pre-incubated under the conditions described in Fig. 1, in the presence of no further additions (■), 5 mM acetate (▲) or 2 μM CCCP (●). After H_2O_2 release rates were stable, different concentrations of P_i were added (as shown) and H_2O_2 release rates relative to control (in the absence of P_i, CCCP or acetate) were plotted. The results shown are representative of 3 similar repetitions.

(Panel B), although the concentrations required for this effect were much larger than those which increased $\Delta\Psi$ (Panel A). The $K_{1/2}$ for the increase in $\Delta\Psi$ was 0.12 mM arsenate, while a half-effect on H_2O_2 release was observed only at 1.81 mM arsenate.

We found that the addition of P_i promoted relative increases in H_2O_2 release under control conditions (Fig. 4, ■) that were only slightly more prominent than those of mitochondria in which ΔpH was eliminated by pre-incubation with acetate (▲). Interestingly, pre-incubation with CCCP (●) completely abolished the P_i effect. Since acetate decreases and CCCP removes the driving force for mitochondrial P_i uptake, this result suggests P_i must be within the mitochondrial matrix to increase mitochondrial H_2O_2 release.

DISCUSSION

We found that P_i uptake can lead to increases in H_2O_2 release from brain, liver and heart mitochondria (Figs. 1, 2A, 4 and results not shown). The changes in H_2O_2 release levels promoted by P_i occurred at constant Δp (Fig. 2D), estimated by measuring $\Delta\Psi$ and ΔpH in parallel (Figs. 2B and C), indicating changes in respiratory rates are not responsible for these effects. Indeed, oxygen consumption was unaltered by P_i under the conditions of Fig. 1 (results not shown).

To investigate a possible link between ΔpH, $\Delta\Psi$ and P_i-induced H_2O_2 release increase, we tested the effect of arsenate and acetate, also capable of increasing $\Delta\Psi$ and collapsing mitocondrial ΔpH. We avoided the use of K^+/H^+ exchanging ionophores to collapse ΔpH due to their ROS scavenging effects.[26] Acetate, which buffers mitochondrial ΔpH (Fig. 2A) but is structurally unrelated to P_i or arsenate, slightly decreased mitochondrial H_2O_2 release (Fig. 2B). Although we have not determined the reason for acetate-induced decreases in mitochondrial H_2O_2 release, we believe it is not directly linked to changes in ΔpH and $\Delta\Psi$ promoted by this anion. Arsenate promoted increases in H_2O_2 release similar to those observed with P_i (Fig. 1). However, arsenate concentrations higher than those which increase $\Delta\Psi$ (Fig. 2A) were required (Fig. 2B). Clearly, ΔpH and H_2O_2 release effects do not have to correlate linearly to be interrelated, so this finding does not eliminate a possible role for ΔpH in the effects of arsenate and P_i. The lack of correlation between the magnitude of the consequences of P_i on $\Delta\Psi$ and H_2O_2 release and those of arsenate may be related to a toxic effect of arsenate. Alternatively, the effects of P_i and arsenate on H_2O_2 release could be ΔpH-independent. Independently of the mechanism through which P_i and arsenate increase mitochondrial ROS, it is clear this release is strongly stimulated by high $\Delta\Psi$, and cannot occur in mitochondria uncoupled by CCCP (Fig. 4).[12,18]

Previous experiments have demonstrated that P_i can increase the probability of mitochondrial permeability transition, a non-selective inner membrane permeabilization caused by excess Ca^{2+} uptake and oxidative stress.[21,27,28] Under these conditions, the presence of Ca^{2+} and P_i can amplify lipid peroxidation reactions by increasing the generation of excited triplet state carbonyls, leading to irreversible mitochondrial damage.[21] Our present results add to this earlier finding, demonstrating that

P_i can lead to increased mitochondrial ROS release even in the absence of supra physiological [Ca^{2+}] and conditions which favor permeability transition. Thus, Ca^{2+} homeostasis does not have to be disrupted for P_i-induced mitochondrial oxidative stress to occur.

The P_i effects on mitochondrial ROS release will surely result in alterations in cellular redox state. By increasing the uptake of mitochondrial P_i, cells may have a substantial increment in the generation of ROS, possibly leading to conditions of oxidative stress. Since intracellular P_i concentrations are expected to rise in a variety of situations in which energy metabolism is hampered, it is possible that mitochondrial oxidative stress secondary to P_i uptake plays a role in cell damage and death under these conditions. Indeed, many pathological states which involve changes in energy metabolism have been linked to mitochondrial oxidative stress.[1–4,9,10] These changes in mitochondrial ROS release have often been attributed to altered respiratory rates, oxygen tensions or depletion of endogenous antioxidants.[1] Our results indicating that increases in P_i concentrations alone are sufficient to significantly augment mitochondrial ROS suggest a further mechanism through which hampered energy metabolism may affect redox state. These findings may contribute toward the understanding of tissue damage in pathological conditions such as ischemia/reperfusion, in which intracellular P_i levels increase due to high-energy phosphate bond hydrolysis.

Acknowledgements

The authors thank Camille C. da Silva and Edson A. Gomes for excellent technical assistance and Professor Roger F. Castilho and Heberty T. F. Facundo for critical reading of the manuscript. This study was supported by *Fundação de Amparo à Pesquisa do Estado de São Paulo* (FAPESP) and *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq). G.A.O. is a student supported by CNPq.

References

[1] Kowaltowski, A.J. and Vercesi, A.E. (1999) "Mitochondrial damage induced by conditions of oxidative stress", *Free Radic. Biol. Med.* **26**, 463–471.
[2] Cadenas, E. and Davies, K.J. (2000) "Mitochondrial free radical generation, oxidative stress, and aging", *Free Radic. Biol. Med.* **29**, 222–230.
[3] Lenaz, G. (2001) "The mitochondrial production of reactive oxygen species: mechanisms and implications in human pathology", *IUBMB Life* **52**, 159–164.
[4] Turrens, J.F. (2003) "Mitochondrial formation of reactive oxygen species", *J. Physiol.* **552**, 335–344.
[5] Vanden Hoek, T.L., Becker, L.B., Shao, Z., Li, C.Q. and Schumacker, P.T. (1998) "Reactive oxygen species released from mitochondria during brief hypoxia induce preconditioning in cardiomyocytes", *J. Biol. Chem.* **273**, 18092–18098.
[6] da Silva, M.M., Sartori, A., Belisle, E. and Kowaltowski, A.J. (2003) "Ischemic preconditioning inhibits mitochondrial respiration, increases H_2O_2 release, and enhances K^+ transport", *Am. J. Physiol.* **285**, H154–H162.
[7] Vanden Hoek, T.L., Becker, L.B., Shao, Z.H., Li, C.Q. and Schumacker, P.T. (2000) "Preconditioning in cardiomyocytes protects by attenuating oxidant stress at reperfusion", *Circ. Res.* **86**, 541–548.
[8] Ferranti, R., da Silva, M.M. and Kowaltowski, A.J. (2003) "Mitochondrial ATP-sensitive K^+ channel opening decreases reactive oxygen species generation", *FEBS Lett.* **536**, 51–55.
[9] Barja, G. (1999) "Mitochondrial oxygen radical generation and leak: sites of production in states 4 and 3, organ specificity, and relation to aging and longevity", *J. Bioenerg. Biomembr.* **31**, 347–366.
[10] Fiskum, G., Starkov, A.A., Polster, B.M. and Chinopoulos, C. (2003) "Mitochondrial mechanisms of neural cell death and neuroprotective interventions in Parkinson's disease", *Ann. N.Y. Acad. Sci.* **991**, 111–119.
[11] Nicholls, D.G. and Ferguson, S.J. (2002) *Bioenergetics 3.* (Academic Press, London).
[12] Korshunov, S.S., Skulachev, V.P. and Starkov, A.A. (1997) "High protonic potential actuates a mechanism of production of reactive oxygen species in mitochondria", *FEBS Lett.* **416**, 15–18.
[13] Korshunov, S.S., Korkina, O.V., Ruuge, E.K., Skulachev, V.P. and Starkov, A.A. (1998) "Fatty acids as natural uncouplers preventing generation of O_2 and H_2O_2 by mitochondria in the resting state", *FEBS Lett.* **435**, 215–218.
[14] Negre-Salvayre, A., Hirtz, C., Carrera, G., Cazenave, R., Troly, M., Salvayre, R., Penicaud, L. and Casteilla, L. (1997) "A role for uncoupling protein-2 as a regulator of mitochondrial hydrogen peroxide generation", *FASEB J.* **11**, 809–815.
[15] Kowaltowski, A.J., Costa, A.D.T. and Vercesi, A.E. (1998) "Activation of the potato plant uncoupling mitochondrial protein inhibits reactive oxygen species generation by the respiratory chain", *FEBS Lett.* **425**, 213–216.
[16] Ruy, F., Vercesi, A.E., Andrade, P.B.M., Bianconi, M.L., Chaimovich, H. and Kowaltowski, A.J. (2004) "A highly active ATP-insensitive K^+ import pathway in plant mitochondria", *J. Bioenerg. Biomembr.* **36**, 195–202.
[17] Murphy, M.P., Echtay, K.S., Blaikie, F.H., Asin-Cayuela, J., Cocheme, H.M., Green, K., Buckingham, J.A., Taylor, E.R., Hurrell, F., Hughes, G., Miwa, S., Cooper, C.E., Svistunenko, D.A., Smith, R.A. and Brand, M.D. (2003) "Superoxide activates uncoupling proteins by generating carbon-centered radicals and initiating lipid peroxidation: studies using a mitochondria-targeted spin trap derived from alpha-phenyl-N-tert-butylnitrone", *J. Biol. Chem.* **278**, 48534–48545.
[18] Skulachev, V.P. (1997) "Membrane-linked systems preventing superoxide formation", *Biosci. Rep.* **17**, 347–366.
[19] Kaplan, R.S., Pratt, R.D. and Pedersen, P.L. (1986) "Purification and characterization of the reconstitutively active phosphate transporter from rat liver mitochondria", *J. Biol. Chem.* **261**, 12767–12773.
[20] Garlid, K.D., Dos Santos, P., Xie, Z.J., Costa, A.D.T. and Paucek, P. (2003) "Mitochondrial potassium transport: the role of the mitochondrial ATP-sensitive K^+ channel in cardiac function and cardioprotection", *Biochim. Biophys. Acta* **1606**, 1–21.
[21] Kowaltowski, A.J., Castilho, R.F., Grijalba, M.T., Bechara, E.J. and Vercesi, A.E. (1996) "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.
[22] Kowaltowski, A.J., Seetharaman, S., Paucek, P. and Garlid, K.D. (2001) "Bioenergetic consequences of opening the ATP-sensitive K^+ channel of heart mitochondria", *Am. J. Physiol.* **280**, H649–H657.
[23] Zhou, M. and Panchuk-Voloshina, N. (1997) "A one-step fluorometric method for the continuous measurement of monoamine oxidase activity", *Anal. Biochem.* **253**, 169–174.

[24] Åkerman, K.E. and Wikstrom, M.K. (1976) "Safranine as a probe of the mitochondrial membrane potential", *FEBS Lett.* **68**, 191–197.
[25] Kowaltowski, A.J., Cosso, R.G., Campos, C.B. and Fiskum, G. (2002) "Effect of Bcl-2 overexpression on mitochondrial structure and function", *J. Biol. Chem.* **277**, 42802–42807.
[26] Grijalba, M.T., Andrade, P.B., Meinicke, A.R., Castilho, R.F., Vercesi, A.E. and Schreier, S. (1998) "Inhibition of membrane lipid peroxidation by a radical scavenging mechanism: a novel function for hydroxyl-containing ionophores", *Free Radic. Res.* **28**, 301–318.
[27] Zoratti, M. and Szabo, I. (1995) "The mitochondrial permeability transition", *Biochim. Biophys. Acta* **1241**, 139–176.
[28] Kowaltowski, A.J., Castilho, R.F. and Vercesi, A.E. (2001) "Mitochondrial permeability transition and oxidative stress", *FEBS Lett.* **425**, 12–15.