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*

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Addition of high concentrations (>1 mM) of inorganic phosphate (P_i) or arsenate to Ca^{2+}-loaded mitochondria was followed by increased rates of H_2O_2 production, membrane lipid peroxidation, and swelling. Mitochondrial swelling was only partially prevented either by butylhydroxytoluene, an inhibitor of lipid peroxidation, or cyclosporin A, an inhibitor of the mitochondrial permeability transition pore. This swelling was totally prevented by the simultaneous presence of these compounds. At lower P_i concentrations (1 mM), mitochondrial swelling is reversible and prevented by cyclosporin A, but not by butylhydroxytoluene. In any case (low or high phosphate concentration) exogenous catalase prevented mitochondrial swelling, suggesting that reactive oxygen species (ROS) participate in these mechanisms. Altogether, the data suggest that, at low P_i concentrations, membrane permeabilization is reversible and mediated by opening of the mitochondrial permeability transition pore, whereas at high P_i concentrations, membrane permeabilization is irreversible because lipid peroxidation also takes place. Under these conditions, lipid peroxidation is strongly inhibited by sorbate, a putative quencher of triplet carboxyl species. This suggests that high P_i or arsenate concentrations stimulate propagation of the peroxidative reactions initiated by mitochondrial-generated ROS because these ions are able to catalyze C_{6n}-aldehyde tautomerization producing enols, which can be oxidized by hemoproteins to yield the lower C_{6n-1}-aldehyde in the triplet state. This proposition was also supported by experiments using a model system consisting of phosphatidylincholine/dicetylphosphate liposomes and the triplet acetone-generating system isobutanal/horseradish peroxidase, where phosphate and Ca^{2+} cooperate to increase the yield of thiobarbituric acid-reactive substances.

The decrease in ATP levels that occurs under pathological conditions such as prolonged anoxia or ischemia/reperfusion results in inorganic phosphate (P_i) accumulation and alterations in intracellular Ca^{2+} homeostasis (1–7). It is argued that high P_i concentrations mimic the metabolic conditions prevalent during ischemia and that, at concentrations higher than 10 mM, P_i inhibits mitochondrial oxidative phosphorylation (8). Indeed, it is well known (see Refs. 9 and 10, and references therein) that accumulation of Ca^{2+} and P_i by mitochondria results in increased permeability of the inner mitochondrial membrane. This is proposed to be a key step in the genesis of cell injury that occurs during ischemia and reperfusion (8–11). Different mechanisms such as lipid peroxidation, phospholipid hydrolysis by phospholipase A_2, or opening of the mitochondrial permeability transition pore (MTP) have been proposed to take place under these conditions (9–14). In this regard, P_i concentration, which is reported to increase up to 20 mM in cells exposed to prolonged anoxia (6), has a profound effect in the rate, extent, and nature of mitochondrial membrane alterations caused by Ca^{2+} ions (8–11, 14–16).

Data from our laboratory (17–20) provided evidence that in the absence of added P_i, the membrane alterations caused by Ca^{2+} seem to be mediated mainly by the attack of mitochondrial generated reactive oxygen radicals (ROS) to membrane proteins, resulting in opening of the MTP, a mechanism sensitive to cyclosporin A (9, 10). This process is stimulated by prooxidants such as t-butyl hydroperoxide or diamide (17, 19, 21), which exhaust mitochondrial reducing power such as NAD(P)H and GSH (9, 22). Under these conditions, the rate of lipid peroxidation is low and the membrane can be quickly resealed by the addition of EGTA and disulfide reductants (17, 21). In contrast, lipid peroxidation is the main form of irreversible mitochondrial membrane permeabilization caused by Ca^{2+} in the presence of other oxidants such as Fe(II)citrate or Fe(II)ATP (23, 24). Interestingly, other data (18, 20) from our laboratory indicate that, while the reduced state of coenzyme Q stimulates the protein oxidation that occurs in the presence of Ca^{2+} and t-butyl hydroperoxide, it protects against damage that occurs in the presence of Ca^{2+} and Fe(II)citrate. This supports the notion that mitochondrial membrane alterations caused by Ca^{2+} in situations of oxidative stress may occur via protein thiol oxidation and/or lipid peroxidation.

The aim of this study was to investigate the mechanisms underlying the stimulation of Ca^{2+}-mediated mitochondrial inner membrane permeabilization by different P_i concentrations. Phosphate is the earliest "inducing agent" described (see

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The abbreviations used are: MTP, mitochondrial permeability transition pore; BHT, butylhydroxytoluene; DCP, dicetylphosphate; HRP, horseradish peroxidase; IBAL, isobutanal; PC, phosphatidylcholine; RLM, rat liver mitochondria; ROS, reactive oxygen species; TBARS, thiobarbituric acid-reactive substances; PUFA, polyunsaturated fatty acid(s).
Mitochondrial Damage Induced by Ca\(^{2+}\) and \(P_i\)

**SCHEME I.** HRP-catalyzed generation of triplet acetone from isobutanal.

Ref. 9, and references therein), and the molecular mechanism by which it mediates membrane permeabilization promoted by Ca\(^{2+}\) remains unclear. Phosphate accumulation, which accompanies intracellular Ca\(^{2+}\) increase during pathological situations, such as prolonged ischemia and reperfusion (see Ref. 8 and references therein), attests to the relevance of this study. Our hypothesis is that high \(P_i\) concentrations and Ca\(^{2+}\) act synergistically increasing the extent of irreversible membrane permeabilization, due mainly to aldehyde \(\alpha\)-peroxidation, which yields triplet carbonyls able to amplify the reaction chain of the peroxidative process (25). This was approached by examining the effect of Ca\(^{2+}\) and phosphate ions on TBARS production by mitochondria and by phosphatidyicholine/dicethylenephosphate (PC/DCP) liposomes challenged with isobutanal (IBAL) (1) as a phosphate-dependent source of the enol (2), which produces triplet acetone (3) in the presence of horseradish peroxidase (HRP) (see Scheme I). Mitochondrial swelling and \(H_2O_2\) production were also monitored in Ca\(^{2+}\) plus phosphate-challenged mitochondria. Inhibition of TBARS production and mitochondrial swelling by sorbate, a quencher of triplet carbonyls (26), supports the proposition that \(P_i\) stimulates lipid peroxidation via aldehyde tautomerization producing enols, very reactive substrates for peroxidases.

**MATERIALS AND METHODS**

Isolation of Rat Liver Mitochondria—Mitochondria were isolated by conventional differential centrifugation from the livers of adult Wistar strain rats fasted overnight. The homogenate was prepared in 250 mM sucrose, 1.0 mM EGTA, and 5.0 mM Hepes buffer, pH 7.2. The mitochondrial suspension was washed twice in the same medium containing 0.1 mM EGTA, and the final pellet was diluted in 250 mM sucrose to a protein concentration of 80–100 mg/ml. These mitochondria contain 8–10 nmol/mg endogenous calcium as determined by atomic absorption spectroscopy.

Liposome Preparation—PC/DCP liposomes were prepared in deionized water by sonication in a Cole-Parmer ultrasonic homogenizer with a nominal output of 100 watts. After 20 min of sonication (with 1-min interval after each 2-min sonication), the solution was centrifuged at 3000 rpm during 5 min. The optical density of the supernatant was determined at 535 nm. In these conditions, the molar extinction coefficient used to calculate TBARS concentrations is 1.56 x 10^

**RESULTS**

Using the classical mitochondrial swelling technique (9), we showed that addition of 4 mM \(P_i\) to a suspension of rat liver mitochondria preincubated (2 min) in reaction medium containing 10 \(\muM\) Ca\(^{2+}\) results in a fast decrease in absorbance of the mitochondrial suspension compatible with extensive mitochondrial swelling (Fig. 1A, line a). The presence of catalase from the beginning of the experiment caused an almost complete inhibition of mitochondrial swelling (line b), suggesting the participation of mitochondrial generated \(H_2O_2\) or \(H_2O_2\)-derived radicals in this process (19). In order to ascertain the nature of the membrane alterations observed under these conditions, experiments were performed in the presence of the antioxidant BHT, an inhibitor of lipid peroxidation, and of cyclosporin A, an inhibitor of MTP opening. Fig. 1 (lines c and d) shows that the mitochondrial swelling was partially prevented either by BHT or cyclosporin A, respectively, and totally prevented by the simultaneous presence of BHT and cyclosporin A (line a). This behavior is compatible with a membrane permeabilization process mediated partly by lipid peroxidation and partly by MTP opening. In contrast, data in the literature (30–32) indicate that mitochondrial swelling induced by Ca\(^{2+}\) in the presence of \(P_i\) can be totally prevented by cyclosporin A. Indeed, Fig. 18 shows that, under our experimental conditions, at lower \(P_i\) concentration (1 mM), cyclosporin A caused an almost complete protection (line a) against membrane permeabilization. The dashed line represents a control experiment in the absence of added phosphate. In this regard, Fig. 2A shows that...
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The mitochondrial swelling induced by 1 mM Pi (Fig. 1B) could be reversed almost completely by the addition of EGTA and ADP, when mitochondria were incubated in a sucrose-free medium. This finding is compatible with membrane resealing (MTP closure), followed by extrusion of the osmotic support that penetrated before addition of EGTA and ADP. At variance with the conditions above, mitochondrial swelling induced by 4 mM Pi was only partially reversed by EGTA and ADP (Fig. 2B), as would be expected if, under these conditions, the membrane permeabilization were caused by both MTP opening and lipid peroxidation.

In order to confirm the occurrence of lipid peroxidation during the mitochondrial swelling induced by high Pi concentrations, the production of thiobarbituric acid-reactive substances (TBARS) was measured. Table I shows that TBARS production by mitochondria incubated in the presence of Pi increases with increasing Pi concentrations. This production was greatly diminished when catalase, EGTA, BHT, or sorbate were present. Accordingly, arsenate, a phosphate analog, was also able to cause a great stimulation of TBARS production. In contrast, acetate, which like Pi or arsenate stimulates Ca²⁺ accumulation by mitochondria by preventing matrix alkalization and elimination of membrane potential, had a much lower effect on lipid peroxidation.

Fig. 3A demonstrates that mitochondrial swelling induced by 4 mM Pi (line d) can be significantly inhibited by sorbate (line b), a putative quencher of triplet carbonyl species (26). This inhibition is enhanced by the simultaneous presence of cyclosporin A (line a), but not of BHT (line c). This suggests that sorbate inhibits only the component of membrane permeabilization related to lipid peroxidation and not by MTP opening. Indeed, Fig. 3B shows that sorbate was not capable of inhibiting swelling induced by 1 mM Pi (line a).

In a previous report we have demonstrated that reduced coenzyme Q is the site for electron leakage responsible for Ca²⁺-induced ROS production in liver mitochondria (20). In order to ascertain the proposition that mitochondrial damage induced by Ca²⁺ plus Pi is mediated by mitochondrial generated ROS at the level of coenzyme Q, we performed experiments with carbonyl cyanide p-trifluorophenylhydrazone (FCCP) and ATP depleted mitochondria treated with antimycin A in the presence or absence of succinate, which maintains coenzyme Q in the reduced or oxidized form, respectively (18). Fig. 4 shows that in the absence of succinate, a condition under which H₂O₂ production is minimal (20), 4 mM Pi addition did not cause mitochondrial swelling (line a), while in the presence of succinate, which increases electron leakage at the level of coenzyme Q (18, 20), a fast and extensive swelling was observed (line d). This swelling was partially inhibited by 100 μM sorbate (line b) or 5 μM BHT (line c) indicating that there is a component of membrane permeabilization related to lipid peroxidation. In addition, Fig. 5 shows that additions of arsenate (line a) or Pi (lines b and c), but not acetate (line d), greatly stimulate the mitochondrial production of H₂O₂ caused by Ca²⁺ alone (line e).

The nature of the synergism between Pi and Ca²⁺ in the process of lipid peroxidation was assessed by using a model system consisting of PC/DCP liposomes and the well known

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**Table I**

| Conditions                  | TBARS formation |
|-----------------------------|-----------------|
| No additions                | 0.30 ± 0.02    |
| 1 mM EGTA                   | 0.16 ± 0.04    |
| 1 mM Pi                     | 0.93 ± 0.03    |
| 2 mM Pi                     | 1.91 ± 0.01    |
| 3 mM Pi                     | 2.24 ± 0.05    |
| 4 mM Pi                     | 2.48 ± 0.07    |
| 5 mM Pi                     | 2.67 ± 0.02    |
| 2.5 mM arsenate             | 2.59 ± 0.05    |
| 20 mM acetate               | 0.91 ± 0.03    |

*Values represent averages of six different experiments ± S.D.*
Mitochondrial Damage Induced by Ca\(^{2+}\) and Pi

**Fig. 4.** Effect of coenzyme Q redox state on mitochondrial swelling induced by Ca\(^{2+}\) and Pi. RLM (0.4 mg/ml) were incubated in reaction medium (125 mM sucrose, 65 mM KCl, 10 mM Hepes buffer, pH 7.2, 5.0 \(\mu\)M rotenone, 2 \(\mu\)M antimycin A, 1 \(\mu\)M carbonyl cyanide p-trifluorophenylhydrazone, and 300 \(\mu\)M Ca\(^{2+}\)) containing: a, no additions; b, 2 mM succinate plus 100 \(\mu\)M sorbate; c, 2 mM succinate plus 5 \(\mu\)M BHT; d, 2 mM succinate. Pi (4 mM) was added where indicated.

**Fig. 5.** Production of \(H_2O_2\) induced by Pi, arsenate, or acetate additions to Ca\(^{2+}\)-loaded mitochondria. RLM (0.3 mg/ml) were added to standard reaction medium containing 1 \(\mu\)M horseradish peroxidase, 1 \(\mu\)M cyclosporin A, and 5 \(\mu\)M BHT. Where indicated by the arrow, 2.5 mM arsenate (a), 4 mM Pi (b), 1 mM Pi (c), or 20 mM acetate (d) was added. Line e represents a control experiment with no additions.

Previous results (17–19) from our laboratory show that mitochondrial membrane permeabilization induced by Ca\(^{2+}\), under conditions of oxidative stress associated with \(t\)-butyl hydroperoxide reduction, is mediated by the oxidative attack of reactive oxygen species to membrane protein thiols. This causes thiol cross-linkage and high molecular weight protein aggregate production that may open a membrane pore upon the binding of Ca\(^{2+}\) (34). These alterations of the inner mitochondrial membrane seem to be related to the state designated as mitochondrial membrane permeability transition, characterized by the reversible opening of a membrane protein pore. The current understanding is that MTP is a protein channel (for review, see Refs. 9 and 10) that is opened by low membrane potential (32, 35), thiol cross-linking agents (34, 36), and various inducing agents in the presence of Ca\(^{2+}\) and closed by EGTA, ADP, Mg\(^{2+}\) (37), sulfhydryl reducing agents (21, 36), protonation of the mitochondrial matrix (32), and submicromolar concentrations of cyclosporin A (30).

This work provides evidence that in the presence of Pi, the increase in membrane permeability caused by Ca\(^{2+}\) can be mediated by alterations of both proteins and lipids. The relat-
The role of Pi seems to be mediated by a stimulation of mitochondrial ROS production at the level of reduced coenzyme Q. Indeed, Kowaltowski et al. provided evidence that, in the case of t-butyl hydroperoxide plus Ca$^{2+}$ (19), Pi plus Ca$^{2+}$ did not cause membrane permeabilization when mitochondria were incubated in the absence of O$_2$. The mechanism by which Pi increases H$_2$O$_2$ production is still unknown but requires the presence of matrix Ca$^{2+}$. The experiments carried out with liposomes (Fig. 6) support the notion that Ca$^{2+}$ binding to inner membrane surface (cardiolipins) decreases negative surface charge density (38) easing the access of Pi to the membrane lipid phase where it may stimulate lipid peroxidation (see Scheme II). This may render the respiratory chain more susceptible to electron leakage and ROS production.

With reference to the mechanisms by which Pi or arsenate stimulate lipid peroxidation, it is important to recall their property to catalyze tautomeration of aldehydes (C$_n$H$_{2n}$O) (25), a process that may be followed by cytochrome-catalyzed peroxidation of the ends formed (Ref. 39 and Scheme II, Reaction 4). This leads to increased production of the C$_n$ - 1 aldehyde in the triplet state (triplet carboxyls), which stimulates lipid peroxidation (Scheme II, Reaction 2b). Lipid peroxidation is a chemiluminescent process, due to the production of triplet carboxyls and singlet oxygen (Scheme II, Reactions 2a and 3a) (40–42).

**Fig. 6. Enhancement of lipid peroxidation by Pi and Ca$^{2+}$ in the model system consisting of PC/DCP 20 mol% liposomes.** Lipid peroxidation was initiated by the triplet acetone-generating system HRP (5 μg)/IBAL (1 mM). PC/DCP liposomes typically contained (PC + DCP) ~ 3.5 mM. The vesicles were incubated with the HRP/IBAL system at 37°C during 20 min, and then samples were taken for TBARS determination, as described under "Materials and Methods." Squares represent the effect of Pi concentration in the presence of 0.5 mM Ca$^{2+}$. Circles represent the effect of Ca$^{2+}$ concentration in the presence of 5 mM Pi.

Triplet carboxyls (Scheme II, Reaction 2b) can initiate the peroxidation of polyunsaturated fatty acids (PUFA) (25) and singlet oxygen (Scheme II, Reaction 3b) directly produces the final product (PUFA-OOH) by 1,3-cycloaddition (42). This would amplify the propagation length of the peroxidative reaction chain. The stimulation by Pi of triplet carbonyl formation is strongly supported by the observed inhibitory effect of sorbate, a triplet carbonyl quencher, on both TBARS yield and mitochondrial swelling. Sorbate has been shown to quench triplet acetone formed by the IBAL/HPR system at the micromolar range (26). Regarding the participation of cytochromes in this process, recent results from our laboratory (39) reveal that mitochondria have the ability to promote a cytochrome-mediated Pi-stimulated oxidation of diphenylcaticatechol. This oxidation produces triplet benzenophene and is followed by a BH$^+$-sensitive process of membrane permeabilization.

In addition, the data presented in this paper is relevant regarding the discrimination of factors determining reversibility versus irreversibility of Ca$^{2+}$-induced cell injury under conditions of ischemia and reperfusion. Although membrane permeabilization determined by MTP opening, in studies in vitro, is easily reversed by pore closure induced by EGTA, disulfide reductants, cyclosporin A, adenine nucleotides, etc. (9) under in vivo conditions, in which phosphate concentrations attain high levels, overlapping of the peroxidative process seems to be an important event leading to irreversible or lethal cell injury.

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