THE ROLE OF Ca^{2+} IN THE PROCESS OF MITOCHONDRIAL DAMAGE ASSOCIATED WITH OXIDATIVE STRESS

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Mitochondria from a wide range of sources have the ability to accumulate Ca^{2+} down their electrochemical gradient mediated by a uniport mechanism and to release the cation via two different pathways: an Na^+-dependent and an Na^+-independent mechanism. The kinetic characteristics of these calcium influx-efflux pathways appear to be incompatible with an important role for mitochondria as cytosolic Ca^{2+} buffers, under resting normal physiological conditions. However under conditions in which a high cytosolic Ca^{2+} concentration is sustained the uniport becomes operative and the matrix Ca^{2+} concentration may attain levels that lead to impairment of mitochondrial functions such as inhibition of oxidative phosphorylation and increase in inner membrane permeability. Accumulation of Ca^{2+} by mitochondria under conditions of oxidative stress induces an increase in inner membrane permeability by a mechanism that appears to be mediated by protein polymerization due to thiol cross-linking.

Keywords: mitochondria; Ca^{2+} transport; oxidative damage; protein thiol cross-linking.

INTRODUCTION

Data from several laboratories[1-3] indicate that disruption of Ca^{2+} homeostasis plays a major role in the pathogenesis of cell injury associated with oxidative stress caused by several toxic agents or pathological states such as ischemia/reperfusion or xenobiotic poisoning. Other data indicate that mitochondrial damage is a crucial step in the sequence of events that lead to cell killing under these conditions[1-5].

Although the involvement of Ca^{2+} in the process of mitochondrial damage caused by oxidizing agents has been extensively demonstrated[6,7], there is no agreement, up to now, regarding this mechanism at the molecular level. The present report summarises data from our laboratory indicating that Ca^{2+} strongly potentiates the toxic effects of reactive oxygen species on isolated mitochondria. An attempt is also made to describe the possible mechanisms underlying these Ca^{2+} effects.

Ca^{2+} TRANSPORT BY MITOCHONDRIA

Mitochondria from a wide range of sources have the ability to accumulate Ca^{2+} down their electrochemical gradient mediated by a uniport mechanism[6,7]. Ca^{2+} influx occurs in response to a negative inside membrane potential that can be developed by respiration, ATP hydrolysis by the F_0F_1-ATPase or the outward flux of K^+ induced by valinomycin[6]. The efflux of the cation is mediated by an Na^+-dependent[8] or an Na^+-independent pathway, proposed to be a $Ca^{2+}/2H^+$ exchanger[9,6]. The simultaneous operation of these two different transport processes for Ca^{2+} uptake and release (i.e., influx-efflux Ca^{2+} cycling) establishes a "set point" for extra mitochoondrial free Ca^{2+} in a range between 0.5 and 1.0 μM[10]. This range of Ca^{2+} concentration is much higher than that normally found in the cytosol under normal physiological conditions[11] and seems to be incompatible with an important role of mitochondria as cytosolic calcium buffers[12]. Instead, the kinetc properties of these Ca^{2+} pathways are compatible with a hypothesis in which the mitochondrial Ca^{2+} transporting system regulates the free Ca^{2+} concentration in the matrix in a range that permits the regulation of three calcium-dependent intramitochondrial dehydrogenases that catalyze rate-limiting reactions of the Krebs cycle (2-oxoglutarate, NAD^+-linked isocitrate and pyruvate dehydrogenases), and stimulate oxidative metabolism and ATP production[12,13]. However, under some pathological conditions the cytosolic Ca^{2+} is sustained at levels compatible with the mitochondrial buffering capacity ($[Ca^{2+}] > 0.5$ μM) and the intramitochondrial Ca^{2+} rises to levels that impair the energy-linked functions of the organelle by at least two independent mechanisms: a) Ca^{2+} competes with Mg^{2+} for the formation of adenine nucleotides complexes[14]. The CaADP-complex, so formed, inhibits oxidative phosphorylation because it competes with MgADP for the active site of the F_0F_1-ATP synthase[15]; b) under oxidative stress conditions, accumulation of Ca^{2+} by mitochondria leads to a condition referred to as "mitochondrial membrane permeability transition", characterized by mitochondrial swelling, loss of the membrane potential and leakiness of the inner membrane to K^+, Mg^{2+}, Ca^{2+}, adenine and pyridine nucleotides and even to protein[6].

Ca^{2+}-DEPENDENT $NADP^+$-INDUCED DAMAGE OF MITOCHONDRIA

It was observed in Prof. Lehninger's laboratory[16,17] that Ca^{2+} efflux from isolated mitochondria could be stimulated by the oxidized state of mitochondrial pyridine nucleotides. This was subsequently confirmed by other laboratories, not only in isolated mitochondria but also in intact cells[4] and perfused liver[18]. It was proposed that this Ca^{2+} release associated with NAD(P)H oxidation and with a low cytosolic phosphorylation potential (Δ Gp), could function as a feedback mechanism to increase the cytosolic phosphorylation potential and the NAD(P)H/NAD(P)+ ratio through the stimulation of cytoplasmic catabolism by the released mitochondrial Ca^{2+} [16]. However, work from our and other laboratories has indicated that this Ca^{2+} efflux was caused by nonspecific increase in membrane permeability[6,7]. Therefore the pyridine nucleotide oxidants were included in the category of agents, referred to as Ca^{2+}-releasing agents, that in conjunction with matrix Ca^{2+} induce the state of membrane permeability transition[19].

The similarity in mitochondrial effects caused by NAD(P)H oxidants and exogenous compounds that generate reactive

oxygen species (ROS)[20,21], led us to propose that the oxidized state of mitochondrial pyridine nucleotides may be associated with an accumulation of ROS, produced by the respiratory chain, in mitochondria exhausted from antioxidants[22].

THE NATURE OF THE MITOCHONDRIAL DAMAGE INDUCED BY CALCIUM PLUS PROOXIDANTS

Fagian et al.[14] presented evidence that the membrane permeability increase of heart submitochondrial particles and liver mitoplasts incubated in the presence of Ca^{2+} and diamide (a -SH oxidant[23]) was associated with the production of protein aggregates due to thiol cross-linking. The production of these aggregates was observed, although at a lesser extent, in liver mitoplasts that accumulated Ca^{2+} in the absence of prooxidants. This suggested that the stimulation in the mitochondrial production of ROS by Ca^{2+} [24,25] could be involved in this mechanism of membrane permeabilization. This would explain the participation of prooxidants in this mechanism by depleting mitochondrial antioxidants such as GSH and NADPH with the consequent increase in mitochondrial ROS concentration. In fact, there are indications that Ca^{2+} and oxidative stress seem to have a concerted action in the mechanism of inner mitochondrial membrane permeabilization[5,24,26]. More recent results have indicated that the oxidative damage of isolated rat liver mitochondria promoted by ROS generated by the metal-catalyzed aerobic oxidation of the 5-aminolevulinic acid was Ca^{2+}-dependent[27] and independent of changes in NAD(P)H redox state[28].

The participation of mitochondrial generated ROS in the mechanism of inner mitochondrial membrane permeabilization by Ca^{2+} plus *t*-butyl hydroperoxide was confirmed by experiments showing that exogenous catalase decreases the production of protein aggregates and prevents the disruption of membrane potential[29]. This protection is certainly due to the decomposition of mitochondrial generated H_20_2 that readily crosses the membrane. This prevents both the accumulation of ROS in the matrix and the oxidation of GSH via H_2O_2 decomposition catalyzed by glutathion peroxidase (see Fig. 1). The protein thiols can be attacked either directly by ROS or indirectly by the products of its attack to polyunsaturated fatty acids, the lipid peroxy or alkoxy radicals[30]. However, the reversibility of the membrane permeabilization process, within short periods of time (up to 10 min), indicates that the extent of lipid peroxidation under these conditions is minimum, otherwise the increase in membrane permeability would be irreversible. This was also confirmed by both the lack of protection conferred by butylhydroxitoluene (inhibitor of lipid peroxidation) and the low extent of TBARS (thiobarbituric acid-reactive substances) production. These results are in line with recent literature indicating that the defect caused by Ca^{2+} plus prooxidants is the consequence of alterations in membrane proteins, rather than in the lipid phase, forming a proteinaceous giant channel sensitive to cyclosporin A, Mg^{2+} and ADP[31,32].

OXIDATIVE DAMAGE OF MITOCHONDRIA INDUCED BY Fe^{2+}/CITRATE IS POTENTIATED BY Ca^{2+} AND INCLUDES LIPID PEROXIDATION AND ALTERATIONS IN MEMBRANE PROTEINS

The nature of the oxidative damage caused in mitochondrial membranes by a free radical generating system containing iron has also been investigated in our laboratory since mobilization of this cation may occur under several pathological conditions[33,34]. Citrate was chosen as iron chelator due to its potential physiological relevance[33] and ability of shifting the Fe^{2+}/Fe^{3+} couple reduction potential from -0.77 V to -0.33 V[35], that results in high rates of Fe^{2+} autoxidation[36]. The results obtained in these studies indicate that the mitochondrial injury caused by Fe^{2+}/citrate was characterized by alterations in membrane proteins and lipid peroxidation. Both type of alterations were stimulated by Ca^{2+}.

At least two types of protein alterations were detected by using SDS-PAGE of the solubilized mitochondrial inner membrane proteins: a) loss of specific protein bands (65 and 116 kDa), which could be recovered from the mitochondrial supernatant; and, b) production of lipid-protein adducts, possibly through Schiff base formation between lipid peroxidation products and amine groups of proteins. The loss of specific proteins may be caused by peroxidation of lipids covalently bound to these membrane proteins[37].

With respect to lipid peroxidation, comparison between the extent of TBARS production in intact mitochondria versus mitoplasts (mitochondria devoid of the outer membrane) indicated that the latter membrane is much more susceptible to this process and to the damaging effects of oxidative stress than the inner membrane. In this regard, data from the literature[38,39] and unpublished results from our laboratory (Castilho, R.F. and Vercesi, A. E.), suggest an important role of reduced ubiquinone in the protection of the inner membrane against the action of lipid radicals. This may also explain the low TBARS production when mitochondria are exposed to Ca^{2+} and prooxidants[29], a condition under which the oxidative stress seems to be restricted to the matrix compartment where the inferred protection by reduced ubiquinone may be facilitated.

POSSIBLE ROLE OF Ca^{2+} IN THE MECHANISM OF INNER MITOCHONDRIAL MEMBRANE PERMEABILIZATION

It can be concluded from the results presented in this paper that Ca^{2+} strongly potentiates the oxidative damage in mitochondria. This role of Ca^{2+} certainly explains the mitochondrial dysfunction associated with the process of cell injury that occurs under oxidative stress, a condition that leads to intracellular Ca^{2+} accumulation[1-3]. It is proposed that oxidative stress increases cytosolyc Ca^{2+} by different mechanisms including the inactivation of Ca^{2+}-ATPases[2,3]. In this regard unpublished data (Castilho RF, Carvalho-Alves PC, Vercesi AE and Ferreira ST) indicate that incubation of sarcoplasmic reticulun vesicles in the presence of Fe^{2+}/H_2O_2/ascorbate leads to alterations in membrane permeability and inactivation of the Ca^{2+}-ATPase. Under *in situ* conditions this process would increase cytosolic Ca^{2+} concentration, exposing mitochondria to its deleterious effects.

The larger question that remains to be answered is the mechanism by which Ca^{2+} potentiates the oxidative damage in mitochondria. On the basis of experimental data, we propose the participation of the cation in at least three steps in the sequence of events that lead to this type of mitochondrial injury (see scheme of Fig.1): i) the protection conferred by exogenous catalase, both in the case of 5-aminolevulinic acid, diamide or *t*-butyl hydroperoxide, strongly suggests the involvement of H_2O_2 in this process. In the presence of Fe^{2+} this compound quickly generates the highly oxidative radical $OH^{\cdot}$. This allows for a proposition connecting the deleterious effects of Ca^{2+} on mitochondria subjected to oxidative stress with results showing the existence of a mitochondrial pool of Fe^{2+}, in liver mitochondria, that can be mobilized by Ca^{2+} accumulation[40]. This is corroborated by data from our group (Kowaltowski, AJ; Castilho, RF and Vercesi, AE., unpublished results) indicating that pre-incubation of mitochondria in the presence of Ca^{2+} and *o*-phenantroline (a Fe^{2+} chelator) causes a time dependent protection against the damaging effects of prooxidants. Accordingly, the accumulation of Ca^{2+} by mitochondria would mobilize Fe^{2+} which stimulates the produc-

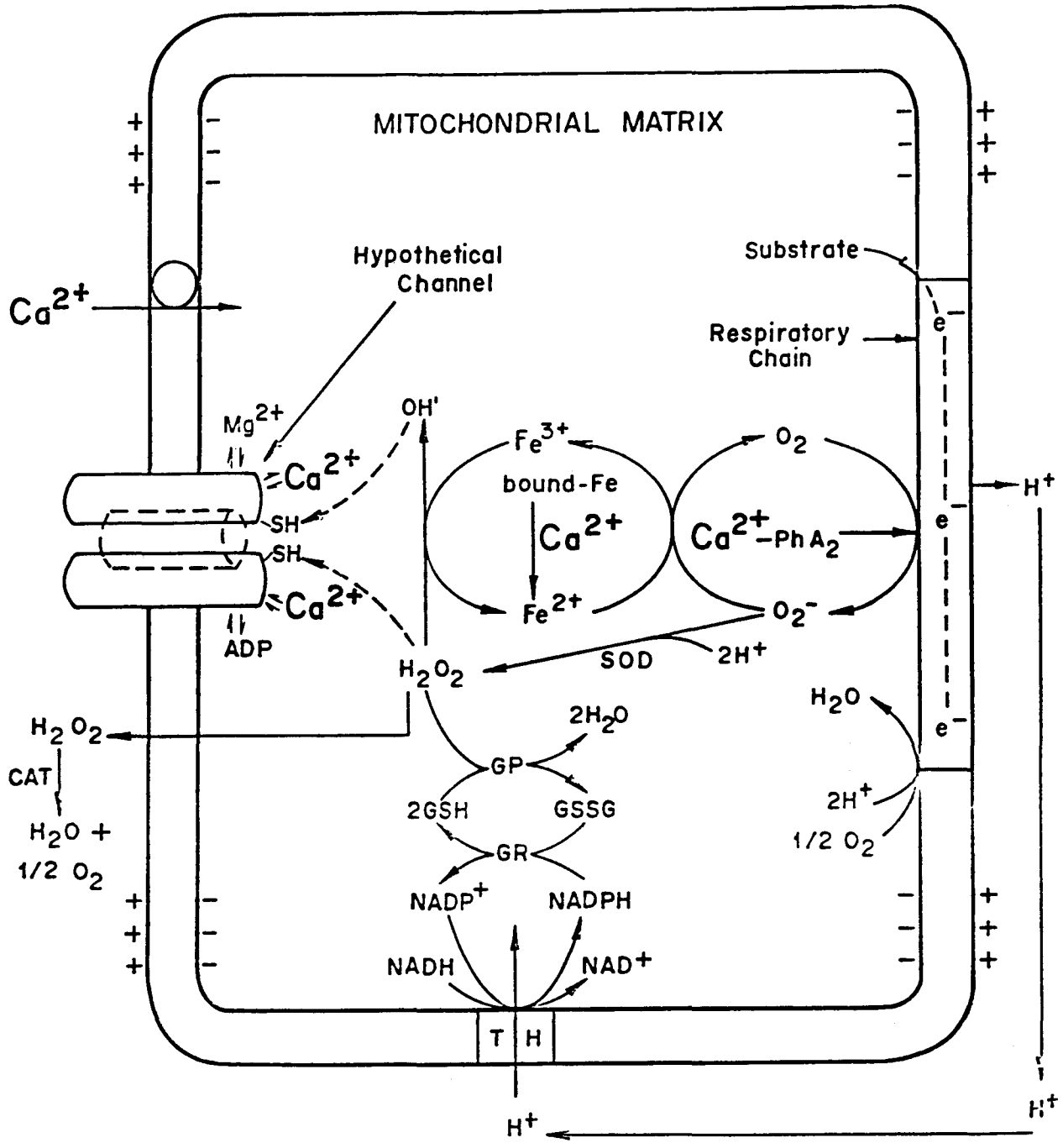

Figure 1. *Scheme summarizing the relationship between the mitochondrial ROS generating and antioxidant defense systems and the hypothetical Ca^{2+}: ROS-induced channel. The respiratory chain generates both ROS ($O_2^{\cdot}$, directly, and H_2O_2 and $OH^{\cdot}$, indirectly) and the electrochemical H^+ potential. The latter can be used by the energy-linked NAD(P)-transhydrogenase (TH) to displace the transhydrogenation reaction in the direction of NADPH production. Under oxidative stress conditions, the lack of reducing equivalents from NAD(P)H and GSH results in accumulation of matrix ROS due to the inefficiency of the reactions catalyzed by glutathion reductase (GR) and glutathion peroxidase (GP). H_2O_2, $OH^{\cdot}$ or other $OH^{\cdot}$ derived radical thus produced may open membrane channels due to the attack against some -SH groups exposed by the binding of Ca^{2+} to critical proteins. These -SH groups may be masked by the binding of ADP or Mg^{2+}, both inhibitors of channel formation. In addition mitochondrial Ca^{2+} may mobilize bound Fe^{2+} and stimulate $O_2^{\cdot}$ production by the respiratory chain due to the activation of the Ca^{2+}-dependent phospholipase A_2 (PhA_2). Exogenous catalase (CAT) also inhibits channel formation, probably via decomposition of mitochondrial H_2O_2 which readily crosses the membrane preventing the accumulation of ROS in the matrix.*

tion of OH· from H_2O_2. The presence of prooxidants exhausts mitochondrial antioxidants such as NAD(P)H and GSH favoring the accumulation of H_2O_2. This mechanism would also explain the activation by Ca^{2+} of the deleterious effects of exogenous Fe^{2+}/citrate without the addition of H_2O_2. This oxidant is produced by mitochondria and the presence of Ca^{2+} would stimulate the Fenton reaction in the matrix due to its effect on mitochondrial Fe^{2+} mobilization; ii) the production of ROS may be augmented by Ca^{2+} via stimulation of the mitochondrial Ca^{2+}-sensitive phospholipase A_2, which would increase the electron leakage at the level of the respiratory chain[24], due to its attack to inner membrane phospholipids ([41]; iii) the binding of Ca^{2+} to some critical membrane protein may cause conformational changes exposing -SH groups to the action of oxidants. This Ca^{2+} effect may be antagonized by Mg^{2+} or ADP.

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