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Inhibition of Mitochondrial Permeability Transition by Low pH is Associated with Less Extensive Membrane Protein Thiol Oxidation

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Ca^{2+} and inorganic phosphate-induced mitochondrial swelling and membrane protein thiol oxidation, which are associated with mitochondrial permeability transition, are inhibited by progressively decreasing the incubation medium pH between 7.2 and 6.0. Nevertheless, the detection of mitochondrial H_2O_2 production under these conditions is increased. Permeability transition induced by phenylarsine oxide, which promotes membrane protein thiol cross-linkage in a process independent of Ca^{2+} or reactive oxygen species, is also strongly inhibited in acidic incubation media. In addition, we observed that the decreased protein thiol reactivity with phenylarsine oxide or phenylarsine oxide-induced swelling at pH 6.0 is reversed by diethyl pyrocarbonate, in a hydroxylamine-sensitive manner. These results provide evidence that the inhibition of mitrochondrial permeability transition observed at lower incubation medium pH is mediated by a decrease in membrane protein thiol reactivity, related to the protonation of protein histidyl residues.

KEY WORDS: Mitochondrial permeability transition; acid pH, protein sulfhydryl oxidation; calcium; reactive oxygen species.

ABBREVIATIONS: DPC, diethyl pyrocarbonate; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); DTT, dithiotreithol; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; MPT, mitochondrial permeability transition; PhAsO, phenylarsine oxide; P_i, inorganic phosphate; RLM, rat liver mitochondria; ROS, reactive oxygen species.

INTRODUCTION

Nonselective inner mitochondrial membrane permeabilization promoted by Ca^{2+} is known as mitochondrial permeability transition (MPT), and can be stimulated by a variety of inducers (for review, see Ref. [1]). These compounds present a large range of chemical characteristics, and include oxidants of pyridine nucleotides, inorganic phosphate (P_i), protonophores and dithiol reagents [1]. We have previously determined that MPT induced by pyridine nucleotide oxidants, protonophores and P_i is

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dependent on reactive oxygen species (ROS) generated by the mitochondrial respiratory chain [2–4]. On the other hand, dithiol reagents such as phenylarsine oxide (PhAsO) can induce MPT even in the absence of mitochondrially-generated ROS, by reacting directly with mitochondrial membrane protein thiol groups [5]. Based on these results, we proposed that ROS-induced MPT occurred secondarily to the oxidation and cross-linkage of membrane protein thiol groups [6]. This was confirmed by the observation of a decrease in mitochondrial membrane protein thiol content and appearance of high molecular weight protein aggregates under conditions leading to MPT [4–6].

MPT impairs mitochondrial function by uncoupling respiration from ATP synthesis when the inner mitochondrial membrane becomes permeable to protons [1]. In fact, MPT occurring in intact cells has been related to necrotic cell death under pathological situations such as ischemia/reperfusion, in which cellular Ca^{2+} homeostasis is compromised [7, 8]. Recently, MPT has also been identified as a crucial event in several experimental models of apoptotic cell death [9, 10].

It has been previously demonstrated that mitochondrial matrix acidification and protonation of membrane protein histidyl residues inhibit MPT [11–14]. This effect may be one of the factors which protects mitochondria in ischemic tissues against MPT [15], since hypoxia is related to an accumulation of lactic acid generated by glycolysis [16]. Supporting this hypothesis, it is known that MPT occurs upon reperfusion [16], when lactic acid is transported out of the cell, resulting in cytosol alkalinization [17]. In this report, we investigate the mechanism by which lowering of incubation medium pH prevents the formation of the MPT pore and consequent nonspecific mitochondrial permeabilization.

MATERIALS AND METHODS

Isolation of Rat Liver Mitochondria

Mitochondria were isolated by conventional differential centrifugation from the livers of adult Wistar rats fasted overnight, as described in Ref. [2].

Standard Incubation Procedure

The experiments were carried out at 28°C, with continuous magnetic stirring. Figs. 1–3(A) were conducted in a standard reaction medium containing 125 mM sucrose, 65 mM KCl, 10 mM MOPS/TRIS buffer, 1 μM antimycin A, 1 μM FCCP, and 1 μM $A_{23187}Ca^{2+}$ ionophore. Other additions are indicated in the figure legends. The results shown are representative or averages $\pm$ s.d. of a series of at least three experiments, using different mitochondrial preparations.

Determination of Mitochondrial Swelling

Mitochondrial swelling was estimated from the decrease in the absorbance of the mitochondrial suspension measured at 520 nm on a Hitachi U-3000 spectrophotometer.

Determination of Mitochondrial H_2O_2 Generation

H_2O_2 production was assessed through the oxidation of scopoletin in the presence of horseradish peroxidase [18]. The decrease in scopoletin fluorescence was monitored at excitation and emission wavelengths of, respectively, 365 and 450 nm, on a Hitachi F-4010 fluorescence spectrophotometer. In these determinations, citrate was used as a Ca^{2+} chelator because EGTA interferes with scopoletin fluorescence. Cyclosporin A was present in all determinations in order to prevent artifacts induced by mitochondrial swelling. Calibration was performed by the addition of known quantities of H_2O_2. No significant difference in calibration was observed in the pH range studied.

Determination of Mitochondrial Membrane Protein Thiol Groups Content

The mitochondrial membrane thiol groups were determined using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, Elmann's reagent) as described in Ref. [4].

Determination of Bovine Serum Albumin (BSA) Thiol Groups Content

300 μl of 1% BSA were incubated for 5 min in the presence of 1 mM DTT in microcon-10^{TM} microconcentrators. The microconcentrators were then centrifuged until all liquid was filtered, and the protein retained was washed twice. The DTT free protein was then resuspended in 300 μl of 10 mM MOPS/TRIS buffer pH 7.2 or 6.0, in the presence or absence of 150 μM PhAsO, 300 μM DPC and 10 mM hydroxylamine. After a 20 min incubation period, the protein was washed again and resuspended in 300 μl of 0.5 M TRIS plus 0.5 mM EDTA, pH 8.3. Total protein content was determined, and 2 μg/ml protein was added to the TRIS/EDTA buffer containing 100 μM DTNB to determine thiol group content. Absorbance was measured at 412 nm.

Chemicals

Antimycin A, Ca^{2+} ionophore A_{23187}, cyclosporin A, DPC, DTNB, FCCP, hydroxylamine, MOPS, P_i, PhAsO, and TRIS were obtained from Sigma Chemical Company (St. Louis, MO,). All other reagents were commercial products of the highest purity grade available.

RESULTS

In order to study mitochondrial permeability transition under distinct pH conditions, we treated mitochondria with the protonophore FCCP, the Ca^{2+} ionophore A_{23187} and the respiratory chain inhibitor antimycin A. This experimental setup eliminates the participation of mitochondrial respiration, membrane potential and Ca^{2+} uptake in the process studied, since these mitochondrial functions may be affected by pH. Under these conditions, Ca^{2+} entrance into the mitochondria can be driven by a concentration gradient across the inner membrane after the addition of a high

concentration of free Ca^{2+} to the extramitochondrial medium [19]. In this experimental situation, when the reaction medium used is at pH 7.2, an extensive decrease in the absorbance of the mitochondrial suspension measured at 520 nm occurs when mitochondria are treated with the MPT inducer P_i (Fig. 1(A), hatched columns), as compared to the suspension not treated with P_i or Ca^{2+} (empty columns), attributed to the nonspecific permeabilization of the inner mitochondrial membrane and mitochondrial swelling. However, this absorbance decrease is less extensive as the reaction medium pH is lowered between 7.2 and 6.0. When MPT was promoted by the dithiol reagent PhAsO, which leads to mitochondrial permeabilization independently of the presence of Ca^{2+} ions or mitochondrially-generated ROS [5, 20], progressively lowering reaction medium pH also prevented the decrease in absorbance of the mitochondrial suspension (full columns). EGTA (100 μM) was present in all experiments studying PhAsO-induced MPT, resulting in a free Ca^{2+} concentration of < 0.01 μM. These results suggest that acidic pH inhibits MPT independently of the presence of Ca^{2+} ions.

Nonspecific mitochondrial permeabilization due to MPT is associated with a decrease in the mitochondrial membrane protein thiol content, which we have previously determined to be due to oxidation and cross-linkage of mitochondrial membrane protein thiol groups [4–6]. In Fig. 1(B), we determined the mitochondrial membrane protein thiol content under conditions similar to those of Fig. 1(A). We observed that the decrease in mitochondrial membrane protein thiol content promoted by Ca^{2+} and P_i (hatched columns) or PhAsO (full columns) is progressively

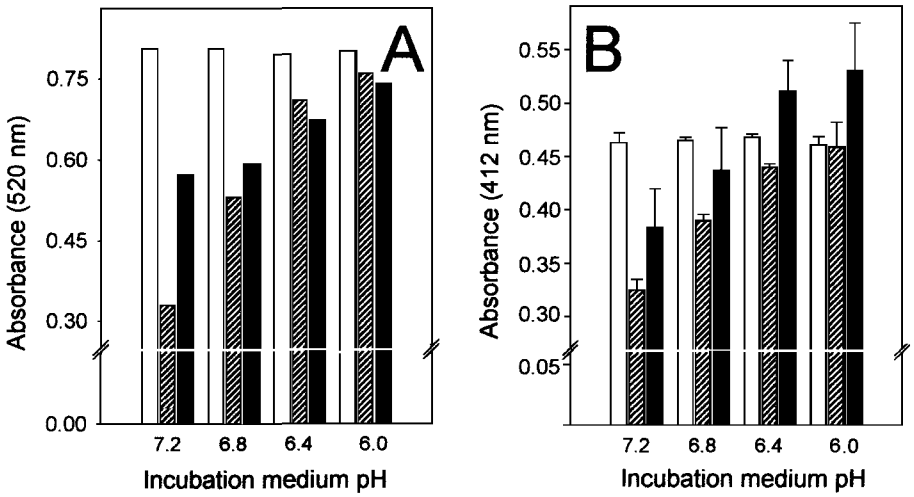

Fig. 1. Effect of medium pH on mitochondrial swelling (Panel A) and membrane protein thiol content decrease (Panel B) induced by Ca^{2+} and P_i or PhAsO. Rat liver mitochondria (RLM—0.3 mg/ml) were incubated for 15 min in standard incubation medium in the presence of no further additions (empty columns), 300 μM Ca^{2+} and 2 mM P_i (hatched columns) or 100 μM EGTA and 150 μM PhAsO (full columns), at the incubation medium pH indicated. The final absorbance of the mitochondrial suspension at 520 nm (Panel A) or membrane protein thiol content (Panel B) were determined as described in Materials and Methods.

inhibited by lowering the incubation medium pH between pH 7.2 and 6.0, in a manner similar to the inhibition of mitochondrial swelling (Fig. 1(A). These results are in agreement with the previous observation that mitochondrial membrane protein thiol reactivity is decreased under lower pH conditions [21]. Medium pH did not have a significant effect on mitochondrial protein thiol content in the absence of MPT inducers (Fig. 1(B), empty columns).

The results in Fig. 1 demonstrate that acidic incubations inhibit both mitochondrial permeabilization and protein thiol content decrease induced by Ca^{2+} and P_i, which is mediated by mitochondrially-generated ROS [4], and by the dithiol reagent PhAsO, which is independent of ROS [5]. This suggests that lowering incubation medium pH does not inhibit MPT by decreasing mitochondrial ROS generation. Indeed, Fig. 2 shows that the detection of Ca^{2+} and P_i-stimulated mitochondrial H_2O_2 generation is progressively *enhanced* by lowering incubation medium pH from 7.2 to 6.0.

Nicolli *et al.* (13) demonstrated that MPT induced by uncouplers could be inhibited by the acidification of the mitochondrial matrix, in a manner sensitive to diethyl pyrocarbonate (DPC), a compound capable of preventing the protonation of various protein residues. Because the effect of DPC could be partially reversed by the addition of hydroxylamine [13, 22], the inhibition of MPT by acidic pH was attributed most probably to the protonation of membrane protein histidyl groups. In Fig. 3(A), we demonstrate that the inhibition of PhAsO-induced MPT at pH 6.0, as measured by mitochondrial swelling (empty columns), can also be reversed by DPC to levels similar to the mitochondrial swelling observed at pH 7.2. The concomitant presence of hydroxylamine partially prevented this effect. Similar results were observed with mitochondrial swelling induced by P_i and Ca^{2+} (*results not shown*). Also, mitochondrial membrane protein thiol content decrease could be observed at pH 6.0 in the presence of DPC, in a manner inhibited by hydroxylamine (Fig. 3(A), full columns). These findings suggest that the protonation of histidyl residues may inhibit mitochondrial membrane protein thiol oxidation, and, in consequence, MPT. In addition, these findings also confirm that low incubation medium pH does not affect the reactivity of PhAsO, since this drug reacted with protein thiols at low pH when in the presence of DPC.

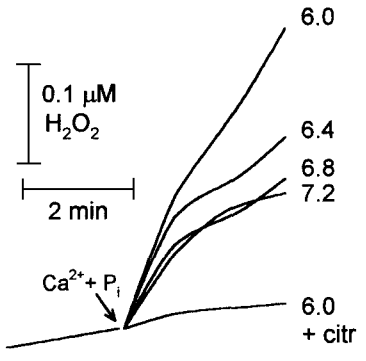

Fig. 2. Mitochondrial generation of H_2O_2 stimulated by Ca^{2+} and P_i-effect of medium pH. RLM (1 mg/ml) were incubated in standard reaction medium containing 1 μM cyclosporin A, 1 μM scopoletin and 0.5 μM horseradish peroxidase at the pH shown. Ca^{2+} (500 μM) and 2 mM P_i were added where indicated. Line "6.0 + citr" represents an experiment conducted in the presence of 100 μM citrate, in which 2 mM P_i was added after 2 min.

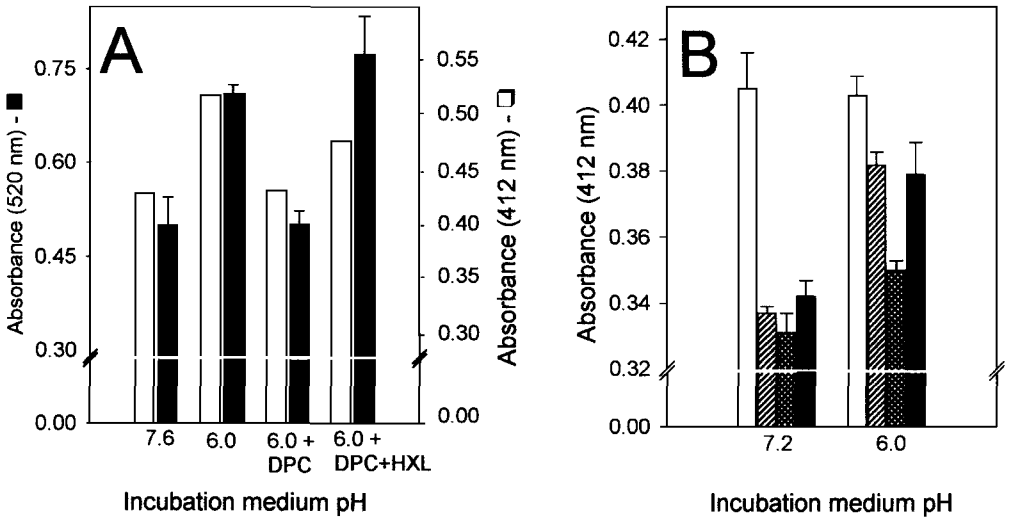

Fig. 3. Effect of DPC and hydroxylamine on PhAsO-induced mitochondrial permeabilization and thiol content decrease (Panel A) or BSA thiol reaction with PhAsO (Panel B). In Panel A, RLM (0.3 mg/ml) were incubated in standard reaction medium containing 150 μM PhAsO and 100 μM EGTA in the pH indicated, in the presence or absence of 300 μM DPC and 10 mM hydroxylamine (HXL), as shown. After 15 min, the absorbance of the mitochondrial suspension at 520 nm (empty columns) or membrane protein thiol content (full columns) were determined. In Panel B, BSA thiol content was determined as described in Materials and Methods. Incubations were conducted at the pH indicated, in the presence of no additions (empty columns), 150 μM PhAsO (hatched columns), 150 μM PhAsO and 300 μM DPC (cross-hatched columns) or 150 μM PhAsO, 300 μM DPC and 10 mM hydroxylamine (full columns).

In Fig. 3(B), we used bovine serum albumin (BSA) as a membrane-free model to study protein thiol reaction with PhAsO, in order to verify if the altered protein thiol reactivity observed at low pH or in the presence of DPC and/or hydroxylamine was related to the conformation of the proteins within the mitochondrial membrane. BSA was pre-treated with DTT to maximize protein thiol reduction, and incubated in the presence of PhAsO at pH 7.2 or 6.0, prior to the determination of the BSA thiol content (see Materials and Methods). We observed that the protein thiol content of BSA (empty columns) decreases more intensely when the protein is treated with PhAsO at pH 7.2 than at pH 6.0 (hatched columns). The concomitant presence of DPC (cross-hatched columns) increased the thiol reaction with PhAsO at pH 6.0 to levels similar to that observed at pH 7.2, in a manner sensitive to hydroxylamine (full columns). Thus, protein thiol reactivity with PhAsO is affected directly by pH, DCP and/or hydroxylamine, even in a membrane-free system.

DISCUSSION

Oxidative alterations of proteins have long been associated with ageing and a large number of diseases including Alzheimer's disease, diabetes and arteriosclerosis (for review, see Ref. [23]). A series of previous reports [4–6] have demonstrated that oxidative alterations of mitochondrial membrane proteins lead to nonspecific

mitochondrial permeabilization, the mitochondrial permeability transition. It is believed that the oxidation and cross-linkage of mitochondrial membrane thiol groups promoted by either mitochondrially-generated ROS or dithiol reagents such as PhAsO leads to the formation of a non-selective inner mitochondrial membrane pore, the MPT pore (for reviews, see Refs. [1, 3]). The opening of the MPT pore results in mitochondrial dysfunction, capable of promoting necrotic cell death by ATP depletion [7, 8]. Also, apoptotic cell death may follow MPT, which results in the release of mitochondrial pro-apoptotic factors, such as cytochrome *c* and the apoptosis inducing factor, from the mitochondrial intermembrane space into the cytosol [9, 10].

In this report, we show that mitochondrially-generated ROS (Fig. 1(B)) or PhAsO (Figs. 1(B) and 3(B)) promote a decrease of mitochondrial membrane protein thiol content in a manner significantly reduced by acidic medium incubations. Interestingly, acidic media increased the detected H_2O_2 generation in deenergized mitochondria stimulated by Ca^{2+} and P_i (Fig. 2). This demonstrates that the presence of ROS, although *necessary* for Ca^{2+} and P_i-induced MPT [2–4], is not a condition *sufficient* to induce protein thiol oxidation and MPT at acidic pH. We have not yet determined the cause for this increased mitochondrial H_2O_2 generation observed in acidic pH media, but it may be related to an enhanced formation of the perhydroxyl radical at the external face of the inner mitochondrial membrane, through the combination of superoxide radicals and protons [24]. The perhydroxyl radical is more diffusable through the inner mitochondrial membrane than the superoxide radical, and permits a greater H_2O_2 production by intramitochondrial superoxide dismutase [24]. In addition, it is important to notice that, in order to eliminate the participation of mitochondrial membrane potential, respiration and Ca^{2+} uptake at different incubation medium pH, all our experiments were conducted in the presence of a respiratory chain inhibitor (antimycin A), a proton ionophore (FCCP) and a Ca^{2+} ionophore (A_{23187}). Indeed, it has been demonstrated that the lowering of incubation medium pH of coupled and respiring mitochondria results in a decrease of mitochondrial membrane potential, Ca^{2+} uptake and ROS generation [25]. These effects may further protect respiring mitochondria from MPT under acidic pH medium conditions.

The prevention of MPT under acidic pH incubations has been demonstrated previously [11–14]. Early studies conducted by Hunter and Haworth [14] suggested that the inhibition of MPT under acidic pH incubations is related to a displacement of Ca^{2+} ions from a Ca^{2+} binding site by protons. This displacement could explain the effect of lower pH on MPT induced by Ca^{2+} and P_i, but we have demonstrated here that acidic pH inhibits also MPT induced by PhAsO in the total absence of Ca^{2+} ions (Fig. 1(B) and 3(A)). Thus, it seems clear that at least another factor must be considered as a cause of MPT inhibition under acidic pH—the decreased membrane protein thiol oxidation.

The reactivity of protein thiols is known to be inhibited in acidic pH. Thiol groups must be in the thiolite form to undergo oxidation, and at acidic pH, the thiolite content of proteins is lower. Thus, it is expected that mitochondrial protein thiol oxidation or reaction with PhAsO is decreased in acidic incubations. The inhibitory effect of low incubation medium pH on protein thiol reaction with PhAsO

could be overcome by the presence of DPC (Figs. 3(A) and (B)), a compound capable of promoting the carbethoxylation of histidyl, tyrosil, sulfhydryl and lysyl protein residues, preventing their protonation [13, 23]. Since the effect of DPC on PhAsO reaction with protein thiol groups was reversed by the concomitant presence of hydroxylamine (Figs. 3(A) and (B)), it is most probable that protein thiol reaction with PhAsO is inhibited in acidic incubations due to the protonation of histidyl residues [23]. Indeed, as demonstrated previously with uncoupler-induced MPT [13], we observed that mitochondrial permeabilization and membrane protein thiol content decrease could be induced by Ca^{2+} and P_i (*results not shown*) or PhAsO (Fig. 3(A)) even at pH 6.0, if DPC was present. Hydroxylamine prevented this DPC effect.

In summary, our results demonstrate a further potential mechanism by which lowering medium pH inhibits mitochondrial permeabilization, namely the prevention of membrane protein thiol oxidation, probably through histidyl residue protonation. This may be one of the main events protecting mitochondria against oxidative damage during acidosis associated with ischemia.

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