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Ascorbate and Low Concentrations of $FeSO_4$ Induce the Ca^{2+}-Dependent Pore in Rat Liver Mitochondria

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Abstract—Oxidative stress is one of the most frequent causes of tissue and cell injury in various pathologies. The molecular mechanism of mitochondrial damage under conditions of oxidative stress induced *in vitro* with low concentrations of $FeSO_4$ and ascorbate (vitamin C) was studied. $FeSO_4$ (1-4 μM) added to rat liver mitochondria that were incubated in the presence of 2.3 mM ascorbate induced (with a certain delay) a decrease in membrane potential and high-amplitude swelling. It also significantly decreased the ability of mitochondria to accumulate exogenous Ca^{2+}. All the effects of $FeSO_4$ + ascorbate were essentially prevented by cyclosporin A, a specific inhibitor of the mitochondrial Ca^{2+}-dependent pore (also known as the mitochondrial permeability transition). EGTA restored the membrane potential of mitochondria de-energized with $FeSO_4$ + ascorbate. We hypothesize that oxidative stress induced *in vitro* with $FeSO_4$ and millimolar concentrations of ascorbate damages mitochondria by inducing the cyclosporin A-sensitive Ca^{2+}-dependent pore in the inner mitochondrial membrane.

Key words: vitamin C, ferrous ion, oxidative stress, permeability transition, cyclosporin A, liver mitochondria

Oxidative stress is a common cause of tissue damage or death in various pathologies [1]. The activation of the so-called pore in the inner mitochondrial membrane with increase in its nonspecific permeability for low molecular weight substances is a possible molecular mechanism of injury to mitochondria during oxidative stress [2, 3]. The pore can be induced by various substances causing oxidative stress, such as pro-oxidants and other substances affecting SH-groups of crucial mitochondrial proteins [4] and some other factors. Several reports [5, 6] suggest that the increase in concentration of reactive oxygen species (ROS) results in pore induction.

Pore opening is often monitored in experiments on isolated mitochondria by the criteria of $\Delta\Psi$ decrease and/or high-amplitude swelling of mitochondria. A specific pore inhibitor, cyclosporin A, prevents both $\Delta\Psi$ decrease and high-amplitude swelling [2].

Ascorbic acid (vitamin C) in the presence of ferrous ions can cause an oxidative stress. Depending on the circumstances, ascorbate can act as either an antioxidant, when it decreases concentrations of toxic ROS forms in cells, or pro-oxidant, when it assists in the formation of extremely toxic hydroxyl radical [7]. Ascorbate effects on mitochondria are of great interest, because this vitamin in high doses is commonly used for prophylactics and therapy, particularly as a cardioprotective antioxidant and for treatment of colds [8-10]. The data of the present study indicates that oxidative stress caused by ferrous ions and millimolar concentrations of ascorbate activates Ca^{2+}-dependent cyclosporin A-sensitive pore(s) in the inner membrane of liver mitochondria.

MATERIALS AND METHODS

Mitochondria were isolated from the livers of white rats (180-200 g) as described previously [11]. Oxygen uptake rate was monitored using an LP7e polarograph equipped with a Clark electrode. Changes in $\Delta\Psi$ level were estimated by the uptake of the potential-sensitive probe safranin O [12] monitored with an Aminco

Abbreviations: EGTA) ethylene glycol-bis(2-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; MOPS) 3-(N-morpholino)-propanesulfonic acid; DNP) 2,4-dinitrophenol; P_i) inorganic phosphate; $\Delta\Psi$) voltage drop across the inner mitochondrial membrane.

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DW2000 spectrophotometer. The difference between optical densities at wavelengths 555 and 523 nm ($\Delta A_{555-523}$) was followed in dual-wavelength mode. The split-beam mode of the Aminco DW2000 spectrophotometer at wavelength 700 nm was used to study the kinetics of swelling of the mitochondria (ΔA_{700} of mitochondrial suspension was recorded).

The incubation medium contained 250 mM sucrose, 5 mM succinate, and 5 mM MOPS-KOH, pH 7.4. Safranin O (12 μM) was added for monitoring ΔΨ. The incubation temperature was 26°C. The mitochondrial suspension was agitated with a magnetic stirrer.

To monitor ΔΨ and swelling kinetics, mitochondria were added to the incubation medium that was intensively stirred in container with a broad bottom. Then, immediately after mitochondria, 4 μM rotenone and 3 mM potassium glutamate were added, and after one minute 2 μg/ml oligomycin was added to the mixture. After 3-4 min of aeration, the mitochondrial suspension was transferred to the spectrophotometer cell where it was agitated with a magnetic stirrer. In most experiments, cyclosporin A was added simultaneously with mitochondria. Other experimental details are given in the figure legends.

Mitochondrial protein was measured by the biuret method.

The following chemicals were used: MOPS, oligomycin, gramicidin D, potassium succinate, fatty-acid-free bovine serum albumin, and potassium glutamate (Sigma, USA); EGTA, rotenone and calcium chloride (Serva, Germany); 2,4-DNP and cyclosporin A (Fluka, Switzerland), $FeSO_4$ and L-ascorbic acid sodium salt (ICN, USA). Sucrose (from a domestic supplier) was recrystallized by ethanol precipitation from its aqueous solution (the ethanol was previously purified by distillation).

RESULTS

$FeSO_4$ (1-2 μM) added to the incubation medium stimulated low oxygen uptake in the presence of 2.3 mM ascorbate even when mitochondria were absent. When mitochondria were added, the oxygen uptake rate increased significantly approaching the maximum uncoupled mitochondrial respiration level. As expected, this increase in oxygen uptake rate was suppressed by EGTA. Short-term incubation (1-2 min) of mitochondria with ascorbate and 2 μM $FeSO_4$ had no effect on either respiratory stimulation by DNP in the presence of EGTA or respiration rate after EGTA and DNP addition; this respiration (in the presence of DNP and EGTA) was completely inhibited by cyanide (data not shown). Thus, the decrease in energy coupling was reversible.

When added simultaneously, 1-2 μM $FeSO_4$ and 2.3 mM ascorbate led (after some lag period) to both ΔΨ decrease and mitochondrial swelling (Fig. 1). In the absence of ascorbate (an equal volume of bidistilled water was added instead), the lag period preceding ΔΨ decrease became significantly prolonged (Fig. 1a, middle curve). The sample containing no ascorbate had virtually no difference from the control containing no ascorbate and $FeSO_4$; ascorbate only had no effect on the lag period duration or decreased it negligibly (data not shown).

A decrease in ΔΨ observed in the presence of both ascorbate and $FeSO_4$ was completely reversed when 100 μM EGTA was added (Fig. 1a, bottom curve). Note that high-amplitude swelling of the mitochondria was also observed in all of these experiments, even when no exogenous P_i was added.

Cyclosporin A, a specific inhibitor of the pore, slightly extended the lag period before ΔΨ decrease in the

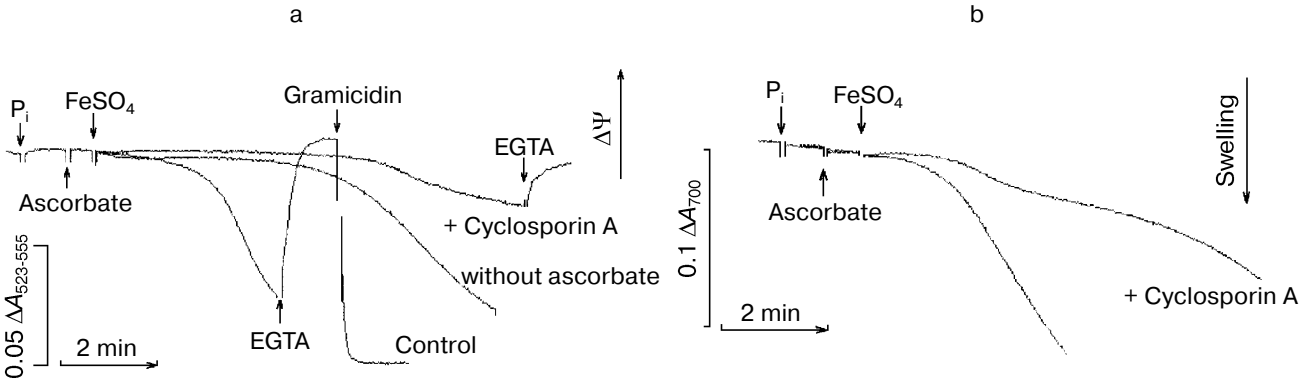

Fig. 1. Induction of the cyclosporin A-sensitive pore in the inner mitochondrial membrane by ascorbate in the presence of ferrous ions. Kinetics of ΔΨ (a) and mitochondrial swelling (b) were monitored after the preincubation of mitochondria (as described in "Materials and Methods") in medium containing 250 mM sucrose, 5 mM succinate, 3 mM glutamate, 5 mM MOPS-KOH (pH 7.4), 4 μM rotenone, and 2 μg/ml oligomycin; 1 μM cyclosporin A was added together with mitochondria. Mitochondrial concentration was 0.8 mg protein per ml. a) The medium also contained 12 μM safranin O. Additives: P_i, 0.1 mM KH_2PO_4; Ascorbate, 2.3 mM ascorbate; $FeSO_4$, 1 μM $FeSO_4$; EGTA, 100 μM EGTA; Gramicidin, 1 μg/ml gramicidin D.

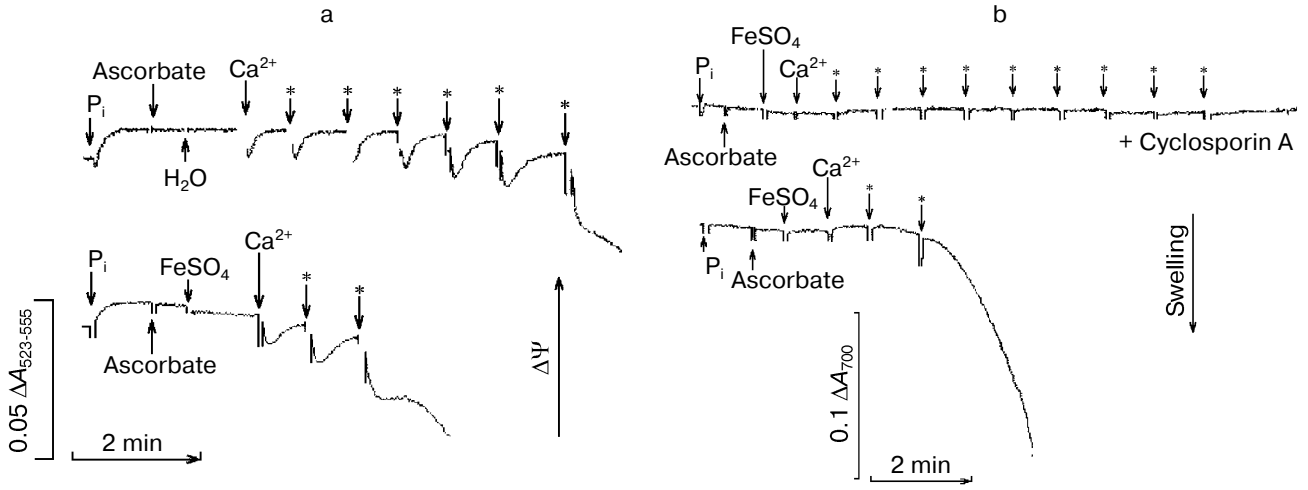

Fig. 2. Effect of ferrous ions on the mitochondrial Ca^{2+}-capacity in the presence of ascorbate. ΔΨ (a) and swelling (b) kinetics were monitored under the same conditions as in Fig. 1. Mitochondrial concentration was 1.7 mg protein per ml. Additives: $FeSO_4$, 4 μM $FeSO_4$; $CaCl_2$, 13 μM $CaCl_2$ (each consecutive portion is indicated by an arrow with an asterisk); other additives were the same as for Fig. 1.

presence of both ferrous ions and ascorbate (Fig. 1a, top curve) and lowered significantly both ΔΨ decrease (Fig. 1a, top curve) and mitochondrion swelling (Fig. 1b, top curve) amplitudes. It is notable that EGTA added in the presence of cyclosporin A terminated completely the drop of ΔΨ and restored its initial level (Fig. 1a, top curve).

When 2.3 mM ascorbate and micromolar $FeSO_4$ were added simultaneously, the mitochondrial calcium capacity decreased significantly in comparison to the sample in which ascorbate only (without $FeSO_4$) was added (Fig. 2a). In this case ΔΨ decrease resulted from the pore opening caused by calcium overload of the mitochondria was followed by their high-amplitude swelling (Fig. 2b, bottom curve). When cyclosporin A and mitochondria were added simultaneously, the calcium capacity of mitochondria increased over the control level (no ascorbate and $FeSO_4$ added), and neither ΔΨ decrease nor mitochondrial swelling was observed during the time of monitoring (Fig. 2b, the top curve).

Interestingly, 0.1 mM P_i added to mitochondria (after addition of oligomycin) led to a slight increase in ΔΨ in some experiments. The lag period between addition of ferrous ion and the beginning of the decrease in ΔΨ was in this case extended (data not shown).

DISCUSSION

At least two different (although related) molecular mechanisms might be responsible for mitochondrial injury under oxidative stress. The peroxidation of the lipids in biological membranes, which is associated with oxidative stress, is well known to affect mitochondrial functions. Peroxidation products inhibit the respiratory chain enzymes [13-15] and increase the permeability of the inner mitochondrial membrane for protons and other ions [16]. It results in to functional impairment and gradual disruption of mitochondria.

Another mechanism that also results in to the mitochondrial injury involves the activation of the inner membrane pore. Lipid peroxidation products may (under certain conditions) potentiate pore opening [2], so both the mechanisms are related to each other. In the presence of ferrous ions, ascorbate may act as a strong inducer of lipid peroxidation [13].

When iron (or other transition metal) reduced by ascorbate enters into Fenton's reaction with hydrogen peroxide, the highly toxic hydroxyl radical is formed, which initiate a chain reaction of peroxidation and can damage mitochondrial proteins [17]. However, it seems that our experimental conditions did not significantly affect the activity of the respiratory chain of mitochondria. A short-term (1-2 min) incubation of mitochondria with ascorbate and 2 μM $FeSO_4$ had no effect on the respiration rate stimulated by the uncoupler 2,4-DNP in the presence of EGTA. Cyanide inhibited completely this respiratory activity. Also, no activation of vigorous mitochondrial lipid peroxidation was observed (mitochondrial lipid peroxidation is accompanied by oxygen uptake that is not inhibited by cyanide or EGTA).

It should be pointed out that ascorbate in the presence of iron activates peroxidation when its concentration is comparatively low (100-660 μM) but inhibits it at higher concentrations (1-5 mM ascorbate in erythrocyte lysate) [18]. Thus, our experimental conditions should not lead to activation of the lipid peroxidation, as it is also evident from the experiments with cyclosporin A. Both

$\Delta\Psi$ drop and mitochondrial swelling under the influence of ascorbate and 1-2 μM $FeSO_4$ were largely prevented or delayed by cyclosporin A, an inhibitor of the mitochondrial pore (Fig. 1). These data unequivocally indicate that the pore of the inner mitochondrial membrane is activated. That cyclosporin A prevents a decrease in calcium capacity in mitochondria induced by ascorbate and $FeSO_4$ (Fig. 2) is also evidence for the involvement of the pores in the mechanism of mitochondrial de-energization by these oxidative stress inducers.

Until now, pore opening in the inner mitochondrial membrane under the influence of ascorbate in the presence of ferrous ions has not been shown. The peculiarities of pore activation and sensitivity to cyclosporin A are known to depend on both the nature of the pore inducer and the experimental conditions [2, 19]. The phenomenology of the pore that is induced by ascorbate plus iron differs from a "classical" permeability transition induced in mitochondria by Ca^{2+} and P_i. However, it is similar to the pore induced in mitochondria by *tert*-butyl hydroperoxide that is commonly used for modeling of oxidative stress effects on mitochondria [19]. Note that the endogenous Ca^{2+} level is sufficient for the opening of the ascorbate/iron-dependent pore.

Recently, it was shown [20] that a certain decrease in both energy coupling and calcium capacity occurs after the incubation of mitochondria in the presence of 100 μM ascorbate and 50 μM ferrous ions with subsequent washing by re-sedimentation. It remains unclear whether the pore opens under these conditions. On the other hand, it is the same low level of ascorbate that matches its level in blood serum when ascorbate is taken in therapeutic doses and particularly together with iron preparations [7].

In conclusion, the effects of ascorbate in cells are still difficult to predict because they depend on the levels of free iron, antioxidants, and many other factors. Iron, particularly its free form, is present in all cells with its highest level in mitochondria [21]. It remains unclear whether a high-vitamin diet may elevate free iron, copper, or cobalt concentrations in cells so that protective antioxidant systems of cells are unable to prevent the damaging pro-oxidative effect of ascorbate. Further studies particularly on cell suspensions and cultures are necessary to understand whether these mitochondrial injuries observed *in vitro* may occur (and under what conditions) *in vivo* when high doses of vitamin C (grams per day [8, 9]) are taken. A pro-oxidative effect of ascorbate in blood serum possibly results in favorable effects such as elimination of bacteria or damaged cells.

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