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Cations SkQ1 and MitoQ Accumulated in Mitochondria Delay Opening of Ascorbate/$FeSO_4$-Induced Nonspecific Pore in the Inner Mitochondrial Membrane

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Abstract—It is known that an addition of $FeSO_4$ in the presence of ascorbic acid to cells or mitochondria can injure energy coupling and some other functions in mitochondria. The present study demonstrates that decrease in ascorbate concentration from 4 to 0.2 mM in the presence of the same low concentrations of $FeSO_4$ accelerates the nonspecific pore opening, while cyclosporin A prevents and under some conditions reverses the pore opening. Hydrophobic cations SkQ1 and MitoQ (structural analogs of plastoquinone and coenzyme Q_{10}, respectively) delay pore opening, SkQ1 being more efficient. It is known that an increase in matrix ADP concentration delays pore opening, while an addition of carboxyatractylate to mitochondria accelerates the beginning of pore opening. Preliminary addition of SkQ1 into a mitochondrial suspension increased the effect of ADP and decreased the effect of carboxyatractylate. These results suggest that under the conditions used SkQ1 protects mitochondria from oxidative damage as an antioxidant when added at extremely low concentrations.

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Injury of the cells under the action of oxidative stress is observed in different pathologies. This can be due to opening of the nonspecific pore for low molecular weight compounds in the inner mitochondrial membrane [1-3]. The opening of the nonspecific pore under the influence of the oxidative stress might be due to an increase in concentration of prooxidants and reactive oxygen species, which trigger chain reactions of peroxidation. This results in oxidation of SH-groups that are crucial for the cell functioning, accumulation of toxic compounds, exhaustion of antioxidant defense systems of the cells, decrease in Ca^{2+}-capacity of mitochondria, and opening of the nonspecific pore. Reactive oxygen species are generated mostly in mitochondria. So, to protect cells from oxidative stress, it is necessary to enhance the antioxidant defense system of mitochondria ([4, 5] and references therein).

During study of mechanisms of mitochondrial damage under the influence of oxidative stress, a peroxidation process is often induced by the addition of $FeSO_4$ together with ascorbate [6]. In experiments with isolated liver mitochondria, the addition of 50 μM $FeSO_4$ with of 0.1 mM ascorbate damaged the mitochondria so severely that they should be first separated from broken mitochondria by centrifugation for further investigation of energy coupling [7]. Previously, we found that the addition of low concentrations (1-3 μM) of $FeSO_4$ in the presence of 5 mM ascorbate resulted in opening of the nonspecific Ca^{2+}-dependent cyclosporin A-sensitive pore in the inner mitochondrial membrane [8].

The principle problem was to induce the nonspecific pore with same characteristics in all sets of experiments with mitochondria isolated from animals derived from different groups during different seasons of the year. The history of discovery of the nonspecific pore is unique. The first publications with the results of ingenious experiments that proved the existence of this pore [9-11] were not understood and were rejected. Only 20 years later, a

Abbreviations: MitoQ) [10-4,5-(dimethyl-3,6-dioxocyclohexa-1,4-dien-1-yl)decyl], compound of ubiquinone and decyltriphenylphosphonium; SkQ1) [10-2-methyl-4,5-(dimethoxy-3,6-dioxocyclohexa-1,4-dien-1-yl)decyl], compound of plastoquinone and decyltriphenylphosphonium; $\Delta\psi$) electric potential difference across the inner mitochondrial membrane.

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sharp increase in the number of similar publications began; some of them repeated the previously described results [12, 13]. This was probably due to strong effects of numerous factors on induction of the pore and to the absence of a specific inhibitor of the activation of the pore in the early investigations.

At present, new antioxidants (hydrophobic cations) have been created that are accumulated in mitochondria via electrophoretic transport according to $\Delta\psi$ (electric potential difference across the inner mitochondrial membrane). Their use is beginning in a number of medico-biological investigations [14, 15]. Effects of low concentrations of SkQ1 [15], a structural analog of plastoquinone containing a positively charged substituting group, and MitoQ [14], a structural analog of coenzyme Q_{10} containing the same positively charged substituting group were investigated in the present study. It is well known that coenzyme Q_{10} can function as an antioxidant in the reduced form but it is a prooxidant in the oxidized form [16]. Since we were interested in the defense of mitochondria from oxidative stress, we studied the effects of these cations on the opening of the pore induced by the addition of $FeSO_4$ in the presence of ascorbate. According to our expectation, SkQ1 and MitoQ should be in the reduced form in such a system, since they are reduced by ascorbate outside mitochondria and by the respiratory chain within mitochondria. It was found in this study that both cations delay the pore opening; SkQ1 was more efficient.

MATERIALS AND METHODS

The following chemicals were used: Mops, oligomycin, rotenone, carboxyatractylate, fat acid-free BSA, succinate, ADP, and $FeSO_4 \cdot 7\ H_2O$ (Sigma, USA); EGTA, safranin O, and KH_2PO_4 (Serva, Germany); cyclosporin A and sodium ascorbate (Fluka, Germany); gramicidin D (MP Biomedicals Inc, USA). SkQ1 and MitoQ were synthesized in the laboratory of V. P. Skulachev [15]. Sucrose was recrystallized twice from a solution in bidistilled water using twice distilled ethanol.

Mitochondria were isolated from rat liver. The isolation medium contained 250 mM sucrose, 5 mM Mops-KOH, pH 7.4, 1 mM EGTA, and BSA (0.5 mg/ml) (here and further in text and figure legends, final concentrations of reagents in incubation media are indicated). The liver was cooled, squeezed through a stainless steel press with holes of 1 mm diameter, and homogenized by hand using a Teflon pestle with a glass (Pyrex) homogenizer. The ratio between medium and tissue weight was approximately 8 : 1. The supernatant after the first centrifugation (10 min, 600*g*) was centrifuged for 10 min at 12,000*g*. The pellet was suspended in 0.5 ml of washing medium (250 mM sucrose, 5 mM Mops-KOH, pH 7.4, and 3 mg/ml BSA) and supplemented with 30 ml of the washing medium but without BSA. The mitochondria were centrifuged again at 12,000*g* for 10 min. The pellet was suspended in 0.02 ml of washing medium with BSA (3 mg/ml). Concentration of mitochondrial protein was determined by the biuret method.

Opening and closing of the pore was studied by recording of $\Delta\psi$ kinetics using the safranin method [17]. The kinetics of the difference in absorption of this potential-sensitive probe between 555 and 523 nm (ΔA) was recorded with an Aminco DW-2000 spectrophotometer in its dual wavelength mode. Succinate, in the presence of rotenone, was used as the oxidation substrate.

Preliminary experiments allowed elaboration of experimental conditions under which low concentrations of SkQ1 exhibited a pronounced protecting effect, i.e. an increase in time interval between the addition of $FeSO_4$ in the presence of ascorbate and the beginning of a decrease in $\Delta\psi$. The incubation medium contained 250 mM sucrose, 5 mM Mops-KOH, pH 7.2, 5 mM succinate, 10 μM safranin O, and BSA (0.1-0.2 mg/ml). Rotenone (2 μM) was added into the sample simultaneously with the mitochondria, and then the sample was supplemented with oligomycin and then with inorganic phosphate. The resulting mixture was supplemented with ascorbate and $FeSO_4$. Simultaneously with ascorbate, the sample was supplemented with an investigated cation dissolved in ethanol, or with the same amount of ethanol as was added with the cation. In some cases, after a pronounced decrease in $\Delta\psi$, the samples were supplemented with 1 μM cyclosporin A, and then an uncoupler gramicidin D was added.

RESULTS AND DISCUSSION

The addition of 3 μM $FeSO_4$ in the presence of 0.2-0.4 mM ascorbate induced faster opening of the nonspecific pore than the addition of the same concentration of Fe^{2+} in the presence of high (4 mM) ascorbate concentrations (data not shown). These results agree with the data obtained previously with lysis of erythrocytes [18]. Increase in ascorbate concentration apparently enhanced both its prooxidant (in the presence of Fe^{2+}) and antioxidant action; probably the first effect increased with ascorbate concentration more slowly than the second.

In the following main experiments, 3 μM $FeSO_4$ (as two consecutive additions of 1.5 μM) was, as a rule, added after ascorbate to induce the nonspecific pore opening. SkQ1 or MitoQ1 (50-120 nM) was added simultaneously with ascorbate. At these concentrations, the penetrating cations delayed opening of the pore, i.e. increased the time interval between the addition of $FeSO_4$ and the beginning of $\Delta\psi$ decrease. When SkQ1 was added simultaneously with ascorbate, the decrease in $\Delta\psi$ was observed significantly later than in the sample without SkQ1 (Fig. 1, compare curves *1* and *4-6*). MitoQ pro-

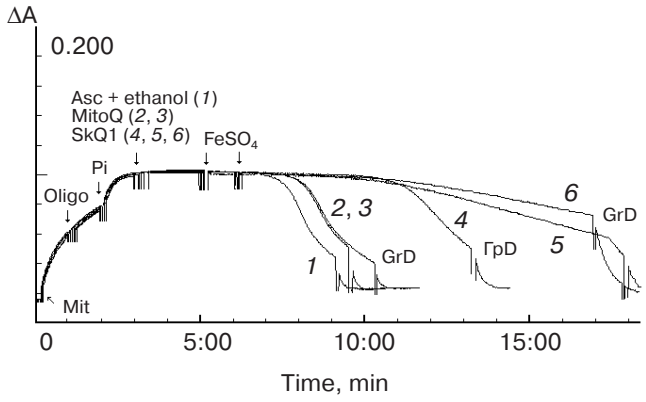

Fig. 1. SkQ1 and MitoQ delay opening of the nonspecific pore with different efficiency. The incubation medium contained 250 mM sucrose, 5 mM Mops-KOH, pH 7.2, 0.2 mg/ml of BSA, 5 mM succinate, 2 μM rotenone, and 10 μM safranin O. Additions: Mit, mitochondria (0.65 mg protein/ml); Oligo, oligomycin (1 μg per mg mitochondrial protein); P_i, 0.1 mM KH_2PO_4; Asc, 0.2 mM ascorbate and ethanol (curve *1*); 60 and 120 nM MitoQ (curves *2* and *3*, respectively); 60, 80, and 120 nM SkQ1 (curves *4-6*, respectively); two additions of 1.5 μM $FeSO_4$; GrD, 2 μM gramicidin D.

duced similar but less pronounced protective effect than SkQ1 (Fig. 1, curves *2* and *3*). Cyclosporin A was not added in the experiments presented in Fig. 1 to observe the Δψ kinetics for longer time.

The data presented in Fig. 2a demonstrate that the addition of cyclosporin A into the incubation medium together with mitochondria completely prevented pore opening induced by ascorbate/$FeSO_4$. Thus, the decrease in Δψ in the experiments is due to activation of the cyclosporin A-dependent nonspecific pore induced by ascorbate and $FeSO_4$. The addition of cyclosporin A after significant decrease in Δψ restored the initial Δψ level (Fig. 2b).

The protective effect of SkQ1 and/or MitoQ was observed in more than 40 assays. Equal concentrations of SkQ1 and MitoQ were added in 18 assays. SkQ1 produced a greater increase in the lag-phase before the pore opening (in the time between the addition of $FeSO_4$ and the beginning of Δψ decrease) in 14 assays. Their efficiency was the same in four assays, and only once both cations accelerated beginning of the pore opening. Thus, the data of the experiments demonstrate that SkQ1 more efficiently protects mitochondria from pore opening induced by ascorbate/$FeSO_4$ compared with MitoQ. To investigate the effect of SkQ1 on the pore, one more approach was used. There is no common opinion concerning the participation of the ATP/ADP antiporter in the protein complex forming the pore [2, 3]. However, there is no doubt that the probability of the opening/closing of the nonspecific pore depends on the conformational state of this anion transporter. The binding of carboxyatractylate to the ATP/ADP antiporter stabilized the *c*-conformation, which promotes pore opening; the same effect is caused by the oxidation of the SH-groups of the antiporter. The binding of ADP to the ATP/ADP antiporter from the matrix side stabilized the *m*-conformation, which prevents pore opening [2, 9-11]. The reversion of pore opening by cyclosporin A (Figs. 2a and 2b) suggests that under the conditions used, the pore opens in a substate with low permeability with "pore flickering" between open and closed states [10, 12, 13].

ADP and then carboxyatractylate were added to mitochondria during beginning of a pronounced decrease in Δψ in the process of the nonspecific pore opening under the described conditions. The addition of ADP increased Δψ almost to the initial level (Fig. 3, curve *1*).

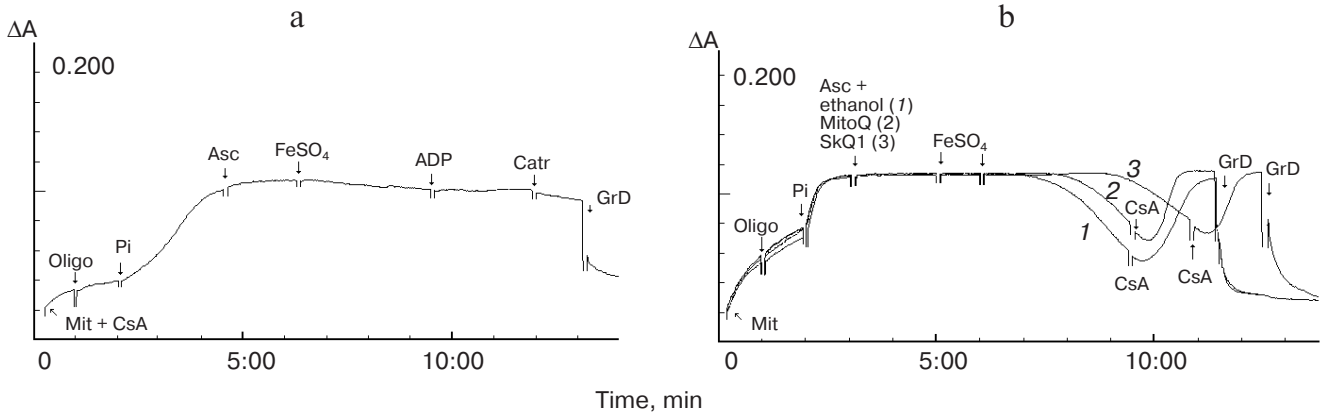

Fig. 2. Cyclosporin A prevents (a) or reverses (b) opening of the nonspecific pore induced by ascorbate/$FeSO_4$. The incubation medium was the same as in the legend to Fig. 1. a) Additions: Mit, mitochondria (0.7 mg/ml); CsA, cyclosporin A (0.4 μg per mg mitochondrial protein); Oligo, oligomycin (1 μg per mg mitochondrial protein); P_i, 0.1 mM KH_2PO_4; Asc, 0.2 mM ascorbate; 3 μM $FeSO_4$; 50 μM ADP; Catr, 2 μM carboxyatractylate; GrD, 2 μM gramicidin D. b) Additions after P_i (0.2 mM KH_2PO_4): Asc, 0.2 mM ascorbate and ethanol (curve *1*); 50 nM MitoQ (curve *2*) or 50 nM SkQ1 (curve *3*); two additions of 1.5 μM $FeSO_4$; CsA, cyclosporin A (0.4 μg per mg mitochondrial protein); GrD, 2 μM gramicidin D.

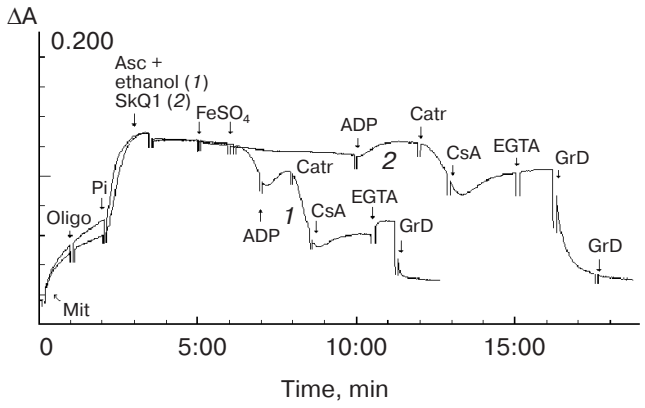

Fig. 3. Effects of ADP and carboxyatractylate on the pore opening induced by $FeSO_4$/ascorbate in the presence of SkQ1 and without it. The incubation medium and additions were the same as in Fig. 1, but after P_i (0.1 mM KH_2PO_4) the sample was supplemented with 0.2 mM ascorbate and ethanol (curve *1*) or 80 nM SkQ1 (curve *2*), then with 2 μM $FeSO_4$, 100 μM ADP, Catr (2 μM carboxyatractylate), CsA (1 μM cyclosporin A), 100 μM EGTA, and GrD (2 μM gramicidin D).

On the contrary, subsequent addition of carboxyatractylate sharply decreased $\Delta\psi$. The addition of 80 nM SkQ1 together with ascorbate decreased the effects (Fig. 3, curve *2*). The results suggest that under the conditions used, SkQ1 protects mitochondria as an antioxidant from the damage caused by opening of the nonspecific pore.

In conclusion, we would like to mention that the hydrophobic antioxidant α-tocopherol is often combined with ascorbic acid in medicine to maintain α-tocopherol in the reduced state for prolonged antioxidant action. In our experiments, ascorbic acid probably reduces SkQ1 and MitoQ outside the mitochondria. However, $FeSO_4$ in the presence of ascorbic acid can damage mitochondria. Under conditions of high content of iron (or other transition metals), it makes sense to replace ascorbic acid with some nontoxic water-soluble antioxidant, or to add the cations with nontoxic chelators.

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REFERENCES

1. Zoratti, M., and Szabo, I. (1995) *Biochim. Biophys. Acta*, **1241**, 139-176.
2. Crompton, M. (1999) *Biochem. J.*, **341**, 233-249.
3. Bernardi, P., Krauskopf, A., Basso, E., Petronilli, V., Blachly-Dyson, E., Di Lisa, F., and Forte, M. A. (2006) *FEBS J.*, **273**, 2077-2099.
4. Papa, S., and Skulachev, V. P. (1997) *Mol. Cell. Biochem.*, **174**, 305-319.
5. Zorov, D. B., Isaev, N. K., Plotnikov, E. Y., Zorova, L. D., Stelmashook, E. V., Vasileva, A. K., Arkhangelskaya, A. A., and Khrjapenkova, T. G. (2007) *Biochemistry (Moscow)*, **72**, 1115-1126.
6. Vladimirov, Yu. A., and Archakov, A. I. (1972) *Peroxide Oxidation of Lipids in Biological Systems* [in Russian], Nauka, Moscow.
7. Schlutter, T., Heinz, S., and Schonfeld, P. (2000) *FEBS Lett.*, **481**, 42-46.
8. Brailovskaya, I. V., Starkov, A. A., and Mokhova, E. N. (2001) *Biochemistry (Moscow)*, **66**, 909-912.
9. Hunter, D. R., and Haworth, R. A. (1979) *Arch. Biochem. Biophys.*, **195**, 453-459.
10. Haworth, R. A., and Hunter, D. R. (1979) *Arch. Biochem. Biophys.*, **195**, 460-467.
11. Haworth, R. A., and Hunter, D. R. (2000) *J. Bioenerg. Biomembr.*, **32**, 91-96.
12. Ichas, F., and Mazat, J.-P. (1998) *Biochim. Biophys. Acta*, **1366**, 33-50.
13. Bodrova, M. E., Brailovskaya, I. V., Efron, G. I., Starkov, A. A., and Mokhova, E. N. (2003) *Biochemistry (Moscow)*, **68**, 391-398.
14. James, A. M., Cocheme, H. M., Smith, R. A., and Murphy, M. P. (2005) *J. Biol. Chem.*, **280**, 21295-21312.
15. Skulachev, V. P. (2007) *Biochemistry (Moscow)*, **72**, 1385-1396.
16. James, A. M., Smith, R. A. J., and Murphy, M. P. (2004) *Arch. Biochem. Biophys.*, **423**, 47-56.
17. Akerman, K. E., and Wikstrom, M. K. (1976) *FEBS Lett.*, **68**, 191-197.
18. Kobayashi, Y., and Usui, T. (1982) *Biochem. Biophys. Res. Commun.*, **105**, 539-545.