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Farnesol-induced generation of reactive oxygen species dependent on mitochondrial transmembrane potential hyperpolarization mediated by F_0F_1-ATPase in yeast

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Abstract An isoprenoid farnesol (FOH) inhibited cellular oxygen consumption and induced mitochondrial generation of reactive oxygen species (ROS) in cells of *Saccharomyces cerevisiae* in correlation with hyperpolarization of the mitochondrial transmembrane potential (mtΔΨ). The FOH-induced events were coordinately abolished with the F_1-ATPase inhibitor sodium azide as well as the F_0F_1-ATPase inhibitor oligomycin, suggesting the dependence of ROS generation on mtΔΨ hyperpolarization mediated by the proton pumping function of F_0F_1-ATPase as a result of ATP hydrolysis. The role of F_1-ATPase activity in mtΔΨ hyperpolarization was supported by the intracellular depletion of ATP in FOH-treated cells and its protection with sodium azide. An indirect mechanism was suggested to exist in the regulation of F_0F_1-ATPase by FOH to accelerate its ATP-hydrolyzing activity.

Key words: Farnesol; Reactive oxygen species; Mitochondrial transmembrane potential; F_0F_1-ATPase; *Saccharomyces cerevisiae*

1. Introduction

An isoprenoid franesol (FOH) is the dephosphorylated form of farnesyl pyrophosphate, which appears as an intermediate of the mevalonate pathway yielding sterols and other isoprenoid compounds [1]. FOH is catabolized into farnesal, farnesoic acid and prenyldicarboxylic acids in mammalian cells [2]. FOH is known to influence the cellular signal transduction when exogenously added and the fact agrees with its rephosphorylation into farnesyl pyrophosphate which also functions as a precursor of protein prenylation [3]. Exposure of cells to isoprenoid such as FOH and geranylgeraniol resulted in apoptosis in various tumor cell lines [4–6], suggesting its relation to inhibition of a phosphatidylinositol type of signaling [6].

In our previous studies [7,8], FOH was found to exhibit a growth inhibitory effect on the yeast *Saccharomyces cerevisiae* by promoting mitochondrial generation of reactive oxygen species (ROS). Although FOH remarkably inhibited the cellular oxygen consumption, none of the direct inhibitory effect was observed with FOH or its possible metabolites on the electron transport of isolated mitochondria. FOH-induced ROS generation was suggested to depend on the interference with a signal transduction which is involved in regulation of mitochondrial electron transport [7]. Protons are pumped out from the mitochondrial matrix into the intermembrane space during electron transport through complex I, III and IV of the respiratory chain. The resulting asymmetrical distribution of protons between the intermembrane space and matrix generates a mitochondrial transmembrane potential (mtΔΨ). Inhibitors of mitochondrial electron transport such as antimycin A and KCN decrease mtΔΨ via inhibition of the proton pumping function of the respiratory chain. Surprisingly, FOH-treated cells were characterized with a greatly increased mtΔΨ, representing a quite unique mechanism for FOH-induced inhibition of mitochondrial electron transport. FOH may interfere with a signaling functional for regulation of mtΔΨ in the yeast cells.

In the present study, we investigated the mechanism of FOH-induced mitochondrial ROS generation in terms of its promoting effect on mtΔΨ hyperpolarization. FOH-induced mtΔΨ hyperpolarization highly depended on the proton pumping function of F_0F_1-ATPase and was considered as a primary cause of mitochondrial ROS generation.

2. Materials and methods

2.1. Strains and media

S. cerevisiae wild-type strain X2180-1A (*MAT*a) and its isogenic [*rho*^{0}] petite mutant [7] were used. Unless otherwise stated, wild-type cells were grown overnight in semi-synthetic lactate medium [9] for preparation of mitochondria and for the assays of cellular oxygen consumption, ROS generation, intracellular ATP content and mtΔΨ. Cells of the petite mutant were grown in YPD medium which contained 1% yeast extract, 2% polypeptone and 2% glucose.

2.2. Assay of ROS generation

Cellular ROS generation was assayed by the method depending on intracellular deacylation and oxidation of 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) to the fluorescent compound 2′,7′-dichlorofluorescein (DCF) [7], except that cells were incubated in 20 mM HEPES buffer (pH 7.4) containing 50 mM glucose.

2.3. Assay of mtΔΨ

After treatment of the cells (10^7/ml) with each chemical in the above buffer, 1 ml of the cell suspension was loaded with 2 μM rhodamine 123 (Rh123) [10,11] for 30 min, washed and resuspended with 100 μl PBS. mtΔΨ was expressed as a fluorescence intensity of Rh123, which was read using a Cytoflow 2300 fluorescence spectrophotometer (Milipore) with excitation at 480 nm and emission at 530 nm.

2.4. Assay of the intracellular ATP content

Cells of [*rho*^{0}] petite mutant were pre-cultured in YPD medium, washed and incubated in 20 mM HEPES buffer (pH 7.4) at a cell

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Abbreviations: FOH, farnesol; ROS, reactive oxygen species; mtΔΨ, mitochondrial transmembrane potential; Rh123, rhodamine 123; FCCP, *p*-trifluoromethoxycarbonylcyanide phenylhydrazone; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; DCF, 2′,7′-dichlorofluorescein; OAG, 1-oleoyl,2-acetyl-*sn*-glycerol; DAG, diacylglycerol

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density of 10^7/ml with or without each inhibitor at 30°C for 30 min. In another experiment using wild-type cells, they were incubated in the same buffer further supplemented with 10 mM 2-deoxyglucose or 10 mM sodium fluoride to prevent glycolytic production of ATP and 3% glycerol for its respiratory production. For the extraction of ATP, cells were collected and mixed with 0.5 ml of 7.5% (w/v) perchloric acid and the mixture was neutralized with 2 M KOH containing 0.5 M triethanolamine as described previously [12]. ATP was measured by the luciferin-luciferase assay according to the method of Doctor et al. [13], using a scintillation counter (Beckman LC 6500) on the single-photon mode.

2.5. Assay of cellular and mitochondrial respiratory activity

The respiratory activity of yeast cells (10^7/ml) was assayed in HEPES buffer (pH 7.4) containing 50 mM glucose by measuring oxygen consumption using a Clark-type oxygen electrode and a Rank Brothers polarograph at 30°C. Yeast mitochondria were isolated from protoplasts according to the method of Glick and Pon [9]. Protein was measured by the method of Bradford [14]. The rate of state 3 oxygen consumption by isolated mitochondria (equivalent to 500 μg protein/ml) was also measured polarographically in 2 mM HEPES buffer (pH 7.4) containing 0.6 M mannitol, 1 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA after supplementation of both 400 μM ADP and 2 mM succinate as respiratory substrates.

2.6. Assay of mitochondrial ATPase activity

Mitochondria (equivalent to 100 μg protein) were pre-incubated in 1 ml of 50 mM Tris-SO_4 buffer (pH 8.5) containing 4 mM $MgSO_4$ with or without inhibitors at 30°C for 5 min. After the addition of ATP at 10 mM, the mitochondrial suspension was further incubated at 30°C for 20 min and inorganic phosphate liberated was determined by the method of Tzagoloff [15]. One unit of the enzyme activity was defined as 1 μmol of ATP hydrolyzed per minute.

2.7. Chemicals

DCFH-DA was a product of Molecular Probe. Other chemicals were purchased from Sigma or of the purest grade commercially available.

3. Results and discussion

3.1. Hyperpolarization of mtΔΨ in FOH-treated cells

As is the case with typical electron transport inhibitors, FOH was likely to reduce mtΔΨ because of its inhibitory effect on mitochondrial electron transport such as reflected by an impairment of cellular oxygen consumption [7]. Thus, we measured mtΔΨ by the uptake of a cationic dye Rh123, the potential-dependent distributional probe. Unexpectedly, Rh123 uptake of the wild-type cells was significantly increased upon FOH treatment in a dose-dependent manner, as shown in Fig. 1A. Rh123 uptake can also be accelerated as a result of mitochondrial swelling in the uncoupler insensitive manner

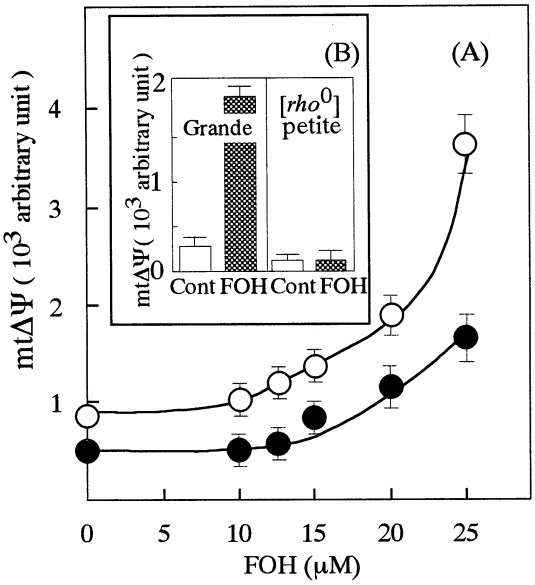

Fig. 1. Promoting effect of FOH on mtΔΨ (A) and its relation to respiration competence (B) in *S. cerevisiae* cells. In (A), mtΔΨ of wild-type cells was measured as a Rh123 fluorescence intensity after FOH treatment at the indicated concentrations in the presence (●) or absence (○) of 20 μM FCCP as described in Section 2. In (B), mtΔΨ was measured as in (A), expect that both wild-type and mutant cells were pre-cultuted in YPD medium. FOH was added at 25 μM for cells of the wild-type grande strain and at 200 μM for cells of the [rho^0] petite mutant strain. Values are means ± S.D. ($n = 5$).

during apoptosis induced by a Fas death signal in SKW6 cells [16]. Unlikely, FOH-induced increase in Rh123 uptake could be protected with the coexistence of *p*-trifluoromethoxycarbonylcyanide phenylhydrazone (FCCP), a potent protonophore that can carry protons across the mitochondrial inner membrane and collapse the proton gradient [17]. This strongly supported the dependence of FOH-induced uptake of Rh123 on the mitochondrial transmembrane proton gradient rather than mitochondrial swelling. Cells of petite mutant cannot generate mtΔΨ by the proton pumping function of the respiratory chain due to the genetic lack of various subunits encoded by mitochondrial DNA [18]. As shown in Fig. 1B, the mutant cells failed to uptake the fluorescent probe even upon treatment with 200 μM FOH, being in agreement with the fact that FOH-induced increase in Rh123 uptake was dependent on hyperpolarization of mtΔΨ but not on the plasma membrane potential. FOH could not induce ROS generation in the

Table 1
Protective effects of mitochondrial ATPase and respiratory inhibitors on FOH-induced mtΔΨ hyperpolarization and ROS generation in *S. cerevisiae*

Addition	mtΔΨ[a] (arbitrary unit)	ROS generation[b] (arbitrary unit)
None	900 ± 69	748 ± 85
FOH (25 μM)	3 702 ± 135	3 848 ± 119
Sodium azide (2 mM)	418 ± 29	650 ± 87
KCN (2 mM)	701 ± 36	687 ± 66
Oligomycin (10 μM)	938 ± 28	668 ± 56
FOH+sodium azide (2 mM)	1 036 ± 122	781 ± 87
FOH+KCN (2 mM)	4 343 ± 187	4 183 ± 205
FOH+oligomycin (10 μM)	1 088 ± 130	698 ± 57

[a]Cells (10^7/ml) were incubated with or without FOH and each inhibitor at 30°C for 30 min. The Rh123 fluorescence intensity was measured as described in Section 2. Values are means ± S.D. ($n = 5$).
[b]After pretreatment with DCFH-DA for 60 min, cells were incubated with or without FOH and each inhibitor as described above. The DCF fluorescence intensity was measured as described in Section 2. Values are means ± S.D. ($n = 5$).

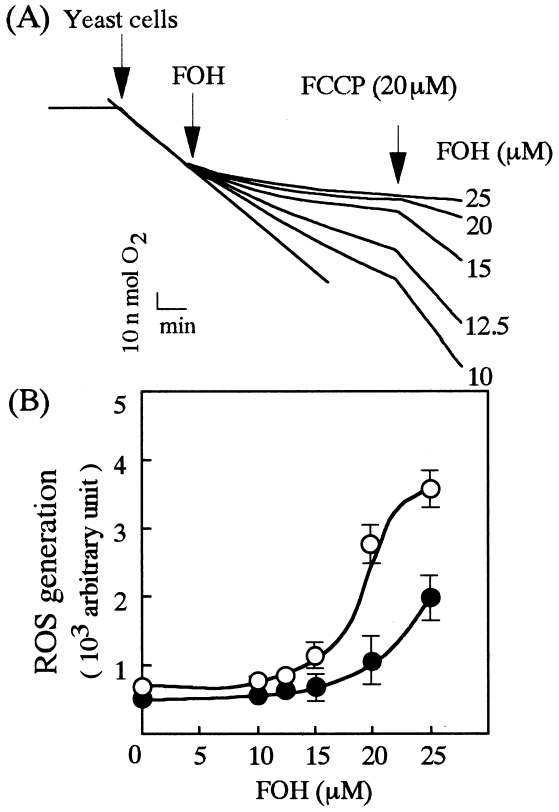

Fig. 2. Protective effects of FCCP on the FOH-induced inhibition of cellular oxygen consumption (A) and ROS generation (B) in the *S. cerevisiae* cells. In (A), the cellular oxygen consumption was monitored as described in Section 2. In (B), ROS generation was assayed using 10^7 cells which were incubated with FOH at the indicated concentrations in the presence (●) or absence (○) of 20 μM FCCP, as described in Section 2. Values are means ± S.D. ($n = 5$).

mutant cells [7], representing the close relation of FOH-induced mitochondrial ROS generation to mtΔΨ hyperpolarization.

3.2. Effects of FCCP on FOH-restricted respiratory activity and FOH-induced ROS generation

In mitochondria, ROS are inevitably produced during electron transfer in the respiratory chain. The electron donor to the molecular oxygen is the unstable ubisemiquinone radical which is generated at complex III in the respiratory chain. ROS generation is multiplied up to the level causing oxidative stress when the mitochondrial electron transport is inhibited by a specific Qi center inhibitor such as antimycin A which accumulates unstable ubisemiquinone radical [19]. ROS are also generated under resting (respiratory control or state 4 respiration) conditions when the electrochemical proton potential difference is high enough so that the rate of electron transport is maximally limited. Under these conditions, cytochromes within the electron transport chain become more reduced and the ubisemiquinone radical becomes long-lived [20]. As shown in Fig. 2A, the cellular oxygen consumption was inhibited with an increasing concentration of FOH up to 25 μM, whereas FOH had a threshold concentration at around 20 μM to achieve sufficient ROS production (Fig. 2B). The extent of ROS production could still be lowered with the coexistence of FCCP even if it was maximally promoted with 25 μM FOH as is the case with FOH-induced hyperpolarization of mtΔΨ (see Fig. 1A). FCCP showed a different type of protective effect on FOH-induced inhibition of cellular oxygen consumption, being ineffective when FOH was added at the threshold concentration for ROS production. These findings revealed a critical role of mtΔΨ for the inhibition of mitochondrial electron transport and thereby evaluated its hyperpolarization as a primary cause of FOH-induced ROS production.

3.3. Protective effects of mitochondrial ATPase and respiratory inhibitors on the FOH-induced hyperpolarization of mtΔΨ

F_0F_1-ATPase consists of two clusters: F_0 sector which functions for proton transport from the intermembrane space to matrix and F_1-ATPase which generally catalyzes ATP synthesis with the aid of the transmembrane proton potential. F_0F_1-ATPase can also mediate the proton pumping function such as transporting protons in the opposite direction across the inner membrane in association with its hydrolytic action on ATP [21]. We therefore examined whether or not FOH-induced hyperpolarization of mtΔΨ depended on asymmetrical proton distribution mediated by the pumping function of F_0F_1-ATPase. Each mitochondrial ATPase and respiratory inhibitor was almost ineffective for ROS generation, as summarized in Table 1. FOH-induced increase in mtΔΨ and ROS generation could be coordinately protected with the coexistence of sodium azide which can inhibit the activity of F_1-ATPase and complex IV [22]. KCN was absolutely ineffective for protection of the FOH-induced events in spite of its inhibitory effect on complex IV. These findings revealed the involvement of F_1-ATPase in hyperpolarization of mtΔΨ and ROS generation. FOH-induced events were also effectively eliminated with oligomycin, which can specifically inhibit F_0F_1-ATPase via binding with F_0 sector [23]. It is most likely that FOH caused mtΔΨ hyperpolarization as a result of proton transport from matrix to the intermembrane space

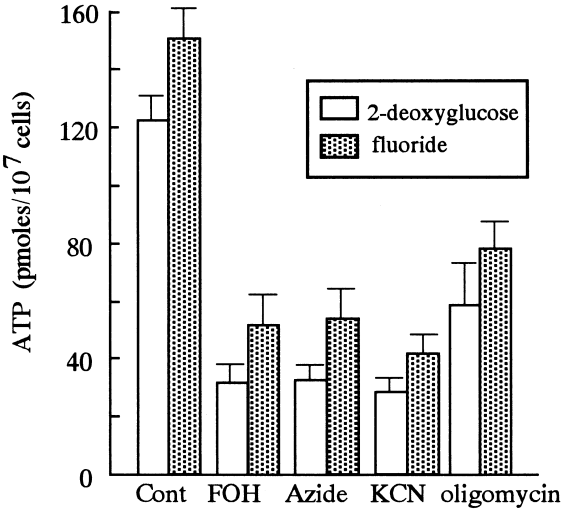

Fig. 3. Effects of respiratory chain inhibitors and FOH on the cellular ATP content under the non-fermentable conditions. The cellular ATP content was measured using 10^7 cells of wild-type strain which were incubated in 20 mM HEPES buffer (pH 7.4) containing 2-deoxyglucose or sodium fluoride as a glycolysis inhibitor, as described in Section 2. Cells were treated with each of 25 μM FOH, 10 μM oligomycin, 2 mM sodium azide or 2 mM KCN at 30°C for 30 min. Values are means ± S.D. ($n = 5$).

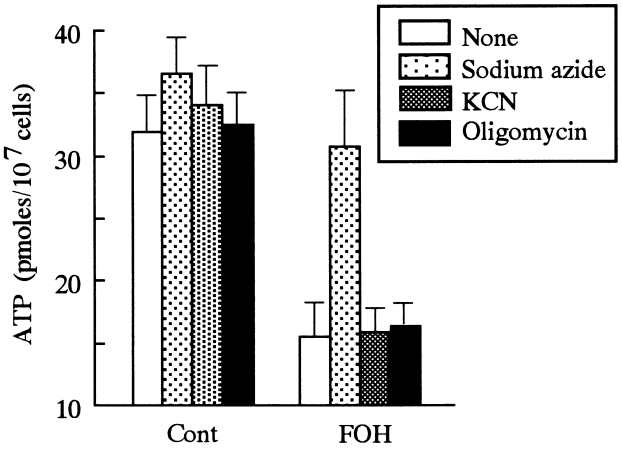

Fig. 4. ATP depletion in FOH-treated cells of [*rho*^{0}] petite mutant and its protection with respiratory inhibitors. The cellular ATP content was measured using 10^7 cells which were incubated in 20 mM HEPES buffer (pH 7.4) with or without 25 μM FOH, as described in Section 2. Cells were pretreated with each of 10 μM oligomycin, 2 mM sodium azide or 2 mM KCN for 10 min prior to the addition of FOH. Values are means ± S.D. ($n = 5$).

through F_0 sector, which was coupled with the hydrolytic reaction of ATP by F_1-ATPase.

3.4. Depletion of ATP in FOH-treated cells

The cellular ATP content should be reduced in response to FOH-induced mt$\Delta\Psi$ hyperpolarization if it was really dependent on the F_1-ATPase activity. However, no difference was observed when cells were treated with FOH in YPD medium which enabled sufficient ATP production by glycolysis (data no shown). In the yeast *S. cerevisiae*, fermentation of glucose has priority over respiratory degradation of this sugar under glucose-rich conditions [24]. In accordance with this fact, respiratory inhibitors such as sodium azide and KCN hardly exhibited any effects on the cellular ATP content (data not shown) when cells were incubated in YPD medium. The effect of FOH on the cellular ATP content was therefore examined in the medium with glycerol as a carbon source for mitochondrial ATP production. As shown in Fig. 3, the cellular ATP content was apparently reduced with each respiratory inhibitor under conditions where 2-deoxyglucose or sodium fluoride was added to absolutely prevent glycolytic ATP production. FOH could also reduce the cellular ATP content but the extent of ATP depletion was kept almost at the same range as those detected with sodium azide or KCN. It was still uncertain whether FOH caused ATP depletion by accelerating F_0F_1-ATPase activity in the ATP-hydrolyzing direction or by simply inhibiting ATP synthesis via oxidative phosphorylation. Cells of petite mutant have a functional F_1-ATPase encoded by nuclear genomes although they lack the respiratory chain in addition to F_0 sector, which is essential for proton transport mediated by F_0F_1-ATPase. The mutant cells maintain their mt$\Delta\Psi$ by a rapid electrogenic exchange of ATP^{4-} for ADP^{3-} with the aid of both F_1-ATPase and an adenine nucleotide translocator [25]. It seems possible to examine the effect of FOH on F_1-ATPase activity with the aid of the mutant cells in which the cellular ATP content is not affected by a respiratory inhibitor. As shown in Fig. 4, FOH was quite effective for reducing the ATP content of the mutant cells. Depletion of ATP could be protected by the co-addition of the F_1-ATPase inhibitor sodium azide but it was not protected with KCN, an inhibitor of complex IV. Oligomycin was also ineffective for the cellular consumption of ATP and the fact was consistent with a loss of the corresponding target such as F_0 sector in the mutant cells. As already described, the mutant cells were absolutely resistant to the action of FOH in terms of ROS generation and mt$\Delta\Psi$ hyperpolarization though they were quite sensitive to the effect of FOH on the cellular ATP level. This agreed with the fact that F_0 sector cannot mediate the proton transport function in the mutant cells due to the genetic lack of subunits 6, 8 and 9, as generally accepted [18]. F_1-ATPase was thus evaluated as a target of FOH as to accelerate the reaction to proceed in favor of ATP hydrolysis and such a regulation could be a primary cause of overall FOH-induced events which critically depended on the proton pumping function of F_0 sector.

FOH did not directly inhibit uncoupler-stimulated mitochondrial oxidase activities [7]. It was still unclear whether or not FOH could directly stimulate F_1-ATPase or inhibit the state 3 respiration coupled with ATP synthesis. As shown in Table 2, sodium azide and oligomycin indeed exhibited the inhibitory activities against both state 3 respiration and ATPase activity of isolated mitochondria. In contrast, FOH did not show any stimulatory or inhibitory effects on both ATPase activity and state 3 respiration even at the concentration of 200 μM. These results were consistent with the previous findings on the failure of FOH in directly inhibiting mitochondrial oxidase activities and suggested the involvement of an indirect mechanism in regulation of F_1-ATPase activity by FOH.

In a human acute leukemia cell line, the apoptotic cell death by the FOH treatment can be protected with the extracellular addition of 1-oleoyl,2-acetyl-*sn*-glycerol (OAG) [5], a membrane-permeable analog of diacylglycerol (DAG) which can activate protein kinase C (PKC). Staurosporine, a potent in-

Table 2
Effects of FOH and mitochondrial ATPase inhibitors on ATPase activity and state 3 respiration of yeast mitochondria

Addition	ATPase activity[a] (U/mg protein)	State 3 respiration[b] (nmol O_2/min/mg protein)
None	0.87 ± 0.08	68.5 ± 4.8
Sodium azide (2 mM)	0.10 ± 0.01	2.3 ± 0.8
Oligomycin (10 μM)	0.19 ± 0.03	12.1 ± 2.9
FOH (25 μM)	0.88 ± 0.07	70.2 ± 6.6
FOH (200 μM)	0.90 ± 0.08	69.2 ± 5.9

[a]Mitochondria (equivalent to 100 μg protein/ml) were incubated with or without each inhibitor at 30°C for 5 min. After addition of ATP, ATPase activity was assayed as described in Section 2. Values are means ± S.D. ($n = 5$).
[b]Mitochondria (equivalent to 500 μg/ml) were incubated with or without each inhibitor at 30°C for 5 min. After addition of both succinate and ADP, state 3 respiration was measured as described in Section 2. Values are means ± S.D. ($n = 5$).

hibitor of PKC, induces mitochondrial ROS generation in mammalian lymphocyte HL-60 cells during the progression of its pro-apoptotic effect [11]. ROS generation is also enhanced in *V-RAS*-transformed mammalian cells [26]. In our previous study [7], the addition of OAG effectively restored FOH-induced ROS generation and the accompanying growth inhibition of *S. cerevisiae* cells. In accordance with this fact, the endogenous DAG content was reduced immediately upon FOH treatment of the yeast cells [8]. FOH-induced growth inhibition of *S. cerevisiae* cells may partly share the same mechanism as is functional for FOH-induced apoptosis of mammalian cells. This is also supported by the fact that the loss of viability upon transformation of the yeast cells with pro-apoptotic Bax was dependent on mitochondrial F_0F_1-ATPase as reflected by the protective effect of oligomycin [27]. In the present study, the mechanism of FOH-induced mitochondrial ROS generation was elucidated in terms of its promoting effect on mt$\Delta\Psi$ hyperpolarization which was mediated by F_0F_1-ATPase. We hereby propose the possibility that FOH can accelerate F_1-ATPase activity to be coupled with the proton pumping function of F_0F_1-ATPase by interfering with cellular signaling.

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