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D-Erythroascorbic acid activates cyanide-resistant respiration in *Candida albicans*

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Abstract

Higher plants, protists and fungi possess cyanide-resistant respiratory pathway, which is mediated by alternative oxidase (AOX). The activity of AOX has been found to be dependent on several regulatory mechanisms including gene expression and posttranslational regulation. In the present study, we report that the presence of cyanide in culture medium remarkably retarded the growth of *alo1*/*alo1* mutant of *Candida albicans*, which lacks D-arabinono-1,4-lactone oxidase (ALO) that catalyzes the final step of D-erythroascorbic acid (EASC) biosynthesis. Measurement of respiratory activity and Western blot analysis revealed that increase in the intracellular EASC level induces the expression of AOX in *C. albicans*. AOX could still be induced by antimycin A, a respiratory inhibitor, in the absence of EASC, suggesting that several factors may act in parallel pathways to induce the expression of AOX. Taken together, our results suggest that EASC plays important roles in activation of cyanide-resistant respiration in *C. albicans*.
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In addition to the conventional cytochrome-involved respiratory pathway, higher plants [1], protists [2] and fungi [3,4] are known to possess an alternative, cyanide-resistant respiratory pathway, which is specifically inhibited by substituted hydroxamic acids [5]. Cyanide-resistant respiration is mediated by alternative oxidase (AOX), which accepts electrons from the ubiquinone pool of the main cytochrome pathway and reduces oxygen to water [6]. The detailed nature and the physiological roles of the alternative respiratory pathway mediated by AOX are still poorly understood. Cyanide-resistant respiration has been shown to be involved in thermogenic inflorescence [7,8], climacteric and ripening of fruits [9], and cell-type proportioning during *Dictyostelium* development [10]. Some reports show that reactive oxygen species, such as superoxide radical anion or hydrogen peroxide, induce the expression of AOX [11,12], suggesting that cyanide-resistant respiration may be related to defense systems against oxidative stress. Recently, AOX from the ascidian *Ciona intestinalis* has been successfully expressed in human cells, providing a useful tool to study the consequences of the respiratory chain dysfunction in animals [13].

In plants, it is common that AOX is encoded by multigene family, whose members are differentially regulated in a tissue- or developmental stage-dependent manner. Three AOX genes have been identified in soybean [14], and two genes in tobacco [15] and rice [16]. In *Arabidopsis thaliana*, the AOX gene family with four members has been characterized [17]. In microorganisms, however, the presence of multigene family for AOX is controversial. It has been reported that AOX is encoded by a single gene in *Trypanosoma brucei* [18] and *Hansenula anomala* [19]. In *Candida albicans*, AOX has been shown to be encoded by a gene family with two members [20,21].

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L-Ascorbic acid (ASC) is produced in all higher plants and in nearly all higher animals except human, other primates, guinea pig, some birds and fish [22]. In animals, a microsomal L-gulono-1,4-lactone oxidase catalyzes the final step of ASC biosynthesis [23,24]. It is believed that the final step of ASC biosynthesis in plants is catalyzed by L-galactono-1,4-lactone dehydrogenase [25]. In some eukaryotic microorganisms, ASC is rare or absent but D-erythroascorbic acid (EASC), a five-carbon analog of ASC, is present [26–30]. The physicochemical properties and biological activities of EASC are very similar to those of ASC [31]. The biosynthetic pathway of EASC from D-arabinose by D-arabinose dehydrogenase and D-arabinono-1,4-lactone oxidase (ALO) has been established in *C. albicans* and *Saccharomyces cerevisiae* [29,30,32,33]. ALO catalyzes the final step of EASC biosynthesis and can also catalyze the production of ASC when L-galactono-1,4-lactone is supplied as a substrate [32,34].

It was reported several decades ago that inhibition of ASC biosynthesis prevents the elicitation of cyanide-resistant respiration in aerated potato tuber slices [35]. However, how ASC biosynthesis is related to the elicitation of cyanide-resistant respiration is still unknown. Here we show that the growth of *C. albicans alo1/alo1* mutant is significantly retarded in the presence of cyanide and the *ALO1* gene is involved in the regulation of AOX expression in *C. albicans*. We also report that increase in the intracellular level of EASC induces the expression of AOX. These results, taken together, suggest that EASC plays important roles in activation of cyanide-resistant respiration in *C. albicans* and raise the possibility that ASC may exert similar effects on cyanide-resistant respiration in higher plants.

Materials and methods

Strains and media. Candida albicans strains used in this study are listed in Table 1. Rich medium (yeast extract/peptone/glucose; YPD) and synthetic complete (SC) medium lacking appropriate supplements for selection were prepared as previously described [36]. Ura$^-$ auxotrophs were selected on SC medium supplemented with 625 mg 5-fluoroorotic acid and 30 mg uridine per liter.

Measurement of respiration. Respiration of cells and isolated mitochondria was measured polarographically at 25 °C using a YSI 5300 Biological Oxygen Monitor Micro System (Yellow Springs Instrument). To induce cyanide-resistant respiration by blocking the conventional cytochrome pathway, 10 μM antimycin A was added to the cell suspension, followed by incubation for 1 h at 28 °C with shaking.

Western blot analysis. Candida albicans mitochondria were isolated as described previously [32]. The purified mitochondria (approximately 30 μg protein) were added to SDS sample buffer (125 mM Tris–HCl, pH 6.8, containing 8% (w/v) SDS, 20% (v/v) glycerol, 0.004% (w/v) bromophenol blue) and boiled for 2 min. 8% polyacrylamide gel was used for separating the mitochondrial proteins. The resolved proteins were transferred onto nitrocellulose membrane and probed with the antibody raised against the AOX protein from *Sauromatum guttatum* [37] at dilutions of 1:1000.

Determination of the intracellular level of hydrogen peroxide. 40 μM 2′,7′-dichlorofluorescin diacetate (Molecular Probes) was added to cells grown to mid-logarithmic phase (5×10^7 cells/ml). After incubation for 30 min at 28 °C, the cells were harvested, washed twice with distilled water, and resuspended at a concentration of 1 g wet cell/ml in 50 mM potassium phosphate, pH 6.5, containing 0.5 mM diethylenetriaminepentaacetic acid. The cells were homogenized with a Mini-Beadbeater (Biospec Products). Unbroken cells and cell debris were removed by centrifugation at 15,000*g* for 10 min. EASC originated from the cells was removed by addition of ascorbate oxidase (Roche) to the resulting supernatant at a concentration of 0.5 U/ml. After incubation for 10 min at 25 °C, the fluorescence of the solution was measured using a SLM Aminco 48000 spectrofluorometer with the excitation at 490 nm and the emission at 526 nm.

Results and discussion

The alo1/alo1 mutant shows growth retardation in the presence of cyanide

To investigate the functional roles of EASC in *C. albicans*, we performed deletion and overexpression of the *ALO1* gene encoding ALO in the previous study [38]. In the WH204 cells with homozygous *alo1/alo1* mutation, the activity of ALO was lost and EASC was not detected at all. In the WH206 cells with a multicopy plasmid containing *ALO1*, the enzymatic activity of ALO and the intracellular EASC level increased up to 3.4-fold and 4.0-fold, respectively, compared with the control WH205 cells. The WH204 cells showed increased sensitivity towards oxidative stress, defective hyphal growth and attenuated virulence [38]. However, the WH204 cells showed no auxotrophy and grew normally in minimal defined medium as well as in rich medium, and their replicative lifespan was indistinguishable from that of the CAI4 cells (data not shown).

It has been reported that inhibition of ASC biosynthesis prevents the elicitation of cyanide-resistant respiration in aerated potato tuber slices [35]. Based on the facts that the physicochemical properties and biological activities of EASC are very similar to those of ASC and *C. albicans* produces EASC instead of ASC, we investigated the possibility whether EASC is involved in the elicitation of cyanide-resistant respiration in *C. albicans*. Interestingly, we found that the presence of cyanide significantly lengthened the lag phase in growth of the WH204 cells (Fig. 1A). The WH206 cells overexpressing *ALO1* showed a shorter lag phase than the control WH205 cells under the same condition (Fig. 1B). These results suggested that EASC is related to the elicitation of cyanide-resistant respiration in *C. albicans*. In log phase and thereafter, however, there was no difference in cell growth pattern between the WH204 and CAI4 cells nor between the WH205 and WH206 cells, indi-

Table 1
Strains used in this study

Strain	Genotype	Source
CAI4	Δ*ura3*::*imm434*/Δ*ura3*::*imm434*	W.A. Fonzi
WH204	Δ*ura3*::*imm434*/Δ*ura3*::*imm434* Δ*alo1*::*hisG*/Δ*alo1*::*hisG*	[38]
WH205	Δ*ura3*::*imm434*/Δ*ura3*::*imm434* (pRC2312)	[38]
WH206	Δ*ura3*::*imm434*/Δ*ura3*::*imm434* (pWK203)	[38]

cating that EASC does not have a significant effect on cell growth once the cells adapt to the presence of cyanide.

EASC activates cyanide-resistant respiration by inducing the expression of AOX

To further investigate the influence of EASC on cyanide-resistant respiration of *C. albicans*, the respiratory activity of *C. albicans* cells was measured with oxygen monitor. The total respiration rate of CAI4 cells was about 108 nmol O_2/h/mg wet cell (Fig. 2A, trace 1). When 1 mM potassium cyanide was added to the cells, the respiration rate decreased to 14 nmol O_2/h/mg wet cell, which corresponds to the rate of cyanide-resistant respiration. The total respiration rate of WH204 cells was a little lower than that of the CAI4 cells and the presence of potassium cyanide completely inhibited the respiration of the WH204 cells (Fig. 2A, trace 2). The total respiration rate of the WH206 cells overexpressing *ALO1* was about 28% higher than that of the control WH205 cells (Fig. 2A, trace 3, 4). Moreover, the respiration rate of the WH206 cells was little changed even under potassium cyanide treatment (Fig. 2A, trace 4). Western blot analysis using the antibody specific for AOX showed that the WH204 cells contained

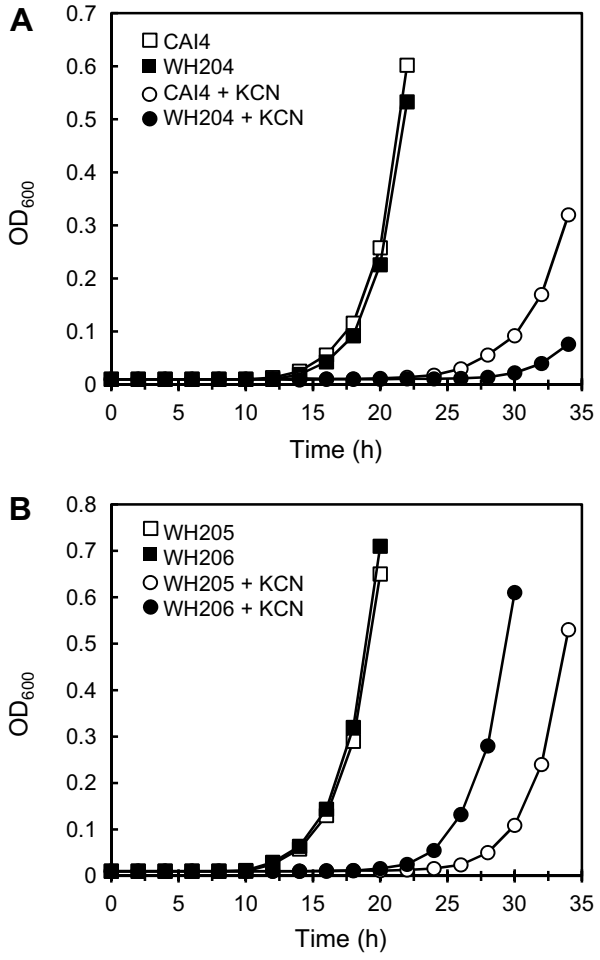

Fig. 1. Deletion and overexpression of *ALO1* affect growth of *C. albicans* in the presence of cyanide. (A) Growth of the control CAI4 and WH204 cells with the *alo1*/*alo1* mutation in the presence or absence of 0.5 mM potassium cyanide. (B) Growth of the control WH205 and WH206 cells overexpressing the *ALO1* gene in the presence or absence of 0.5 mM potassium cyanide. Cells were grown at 28 °C in SC medium with or without 0.5 mM potassium cyanide. Cell growth was monitored by measuring the optical density at 600 nm.

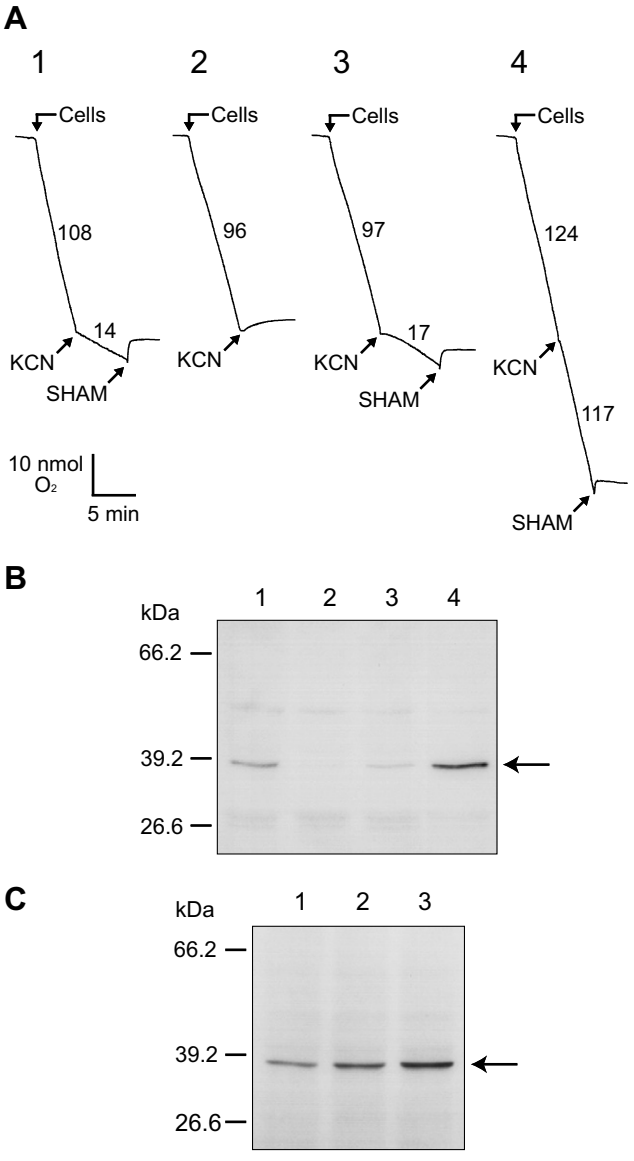

Fig. 2. EASC activates cyanide-resistant respiration by inducing the expression of AOX in *C. albicans*. (A) Measurement of respiratory activity of fresh *C. albicans* cells. Trace 1, CAI4; trace 2, WH204; trace 3, WH205; trace 4, WH206. Rates on the respiration traces are in nmol O_2/h/mg wet cell. Respiratory inhibitors, 1 mM potassium cyanide (KCN) and 2 mM salicylhydroxamic acid (SHAM), were added as indicated by arrows. (B) Western blot analysis of AOX from *C. albicans*. Mitochondria (approximately 30 μg protein) from the CAI4 (lane 1), WH204 (lane 2), WH205 (lane 3) and WH206 cells (lane 4) were probed with the antibody against AOX. Arrow indicates AOX. (C) Western blot analysis of AOX from the CAI4 cells incubated for 2 h at 28 °C in 50 mM potassium phosphate buffer, pH 6.5, 0.1 M glucose (lane 1), containing 10 mM D-arabinono-1,4-lactone (lane 2) or 10 mM EASC (lane 3). Arrow indicates AOX.

little, if any, AOX protein (Fig. 2B). In contrast, the expression level of AOX increased significantly in the WH206 cells compared with the control WH205 cells, correlating well with the measurement of respiration. These results indicated that EASC is an important factor in regulating the expression of AOX in *C. albicans*.

Exogenous addition of D-arabinono-1,4-lactone, the physiological substrate of ALO, can increase the intracellular level of EASC [32,34]. To examine whether induction of the AOX expression is caused by increase in the intracellular level of EASC or by that of ALO protein, we added D-arabinono-1,4-lactone or EASC exogenously to *C. albicans* cells and analyzed the expression level of AOX. Western blot analysis showed the protein level of AOX remarkably increased when D-arabinono-1,4-lactone or EASC was exogenously added to the CAI4 cells (Fig. 2C). ALO can also catalyze the production of ASC efficiently when L-galactono-1,4-lactone is supplied as a substrate [32,34]. We could observe a similar effect on the AOX expression by exogenous addition of L-galactono-1,4-lactone or ASC (data not shown). These results confirmed that the increased intracellular level of EASC, not ALO protein, induces the expression of AOX in *C. albicans*. Moreover, induction of the AOX expression by ASC in *C. albicans* raised the possibility that ASC may regulate the AOX expression also in higher plants.

Hydrogen peroxide, a by-product of the enzymatic reaction catalyzed by ALO, has been reported to increase the expression level of AOX [12,39]. To test the possibility whether the changes in expression of AOX in the WH204 and WH206 cells might be mediated by hydrogen peroxide, we measured the intracellular level of hydrogen peroxide in the cells by fluorometric assay using 2′,7′-dichlorofluorescin diacetate. There was not a significant difference in the intracellular level of hydrogen peroxide between the WH204 and CAI4 cells. The intracellular level of hydrogen peroxide in the WH206 cells was also similar to that in the control WH205 cells (data not shown). These observations confirmed that regulation of the AOX expression is mediated by EASC, not by hydrogen peroxide, in *C. albicans*.

AOX can be induced by antimycin A in the alo1/alo1 mutant

It has been reported that the respiratory inhibitors that block the conventional cytochrome pathway can induce the expression of AOX [40,41]. In accordance with these reports, incubation in the presence of 10 μM antimycin A greatly increased the rates of cyanide-resistant respiration in the CAI4 cells, and even in the *alo1/alo1* WH204 cells as well (Fig. 3A, trace 1, 2). However, the cyanide-resistant respiration rate of antimycin A-treated WH204 cells was about 49% lower than that of the CAI4 cells. When incubated with antimycin A, the WH206 cells overexpressing *ALO1* showed about 2.0-fold increase in the cyanide-resistant respiration rate compared with the control WH205 cells (Fig. 3A, trace 3, 4). These results indicated that AOX can still be induced in the absence of EASC and suggested that several factors may act in parallel pathways to induce the expression of AOX.

Interestingly, although the cyanide-resistant respiration rate under antimycin A treatment was different between the CAI4 and WH204 cells or between the WH205 and WH206 cells, the expression levels of AOX were similar among all the cells (data not shown). These results raised the possibility that EASC may stimulate the respiratory activity of *C. albicans* cells by direct participation in electron transport. Supporting this possibility, when the respiratory activity of mitochondria isolated from the CAI4 cells was measured, the respiration rate dramatically increased upon addition of EASC; the respiration rate of mitochondria in the presence of cyanide was

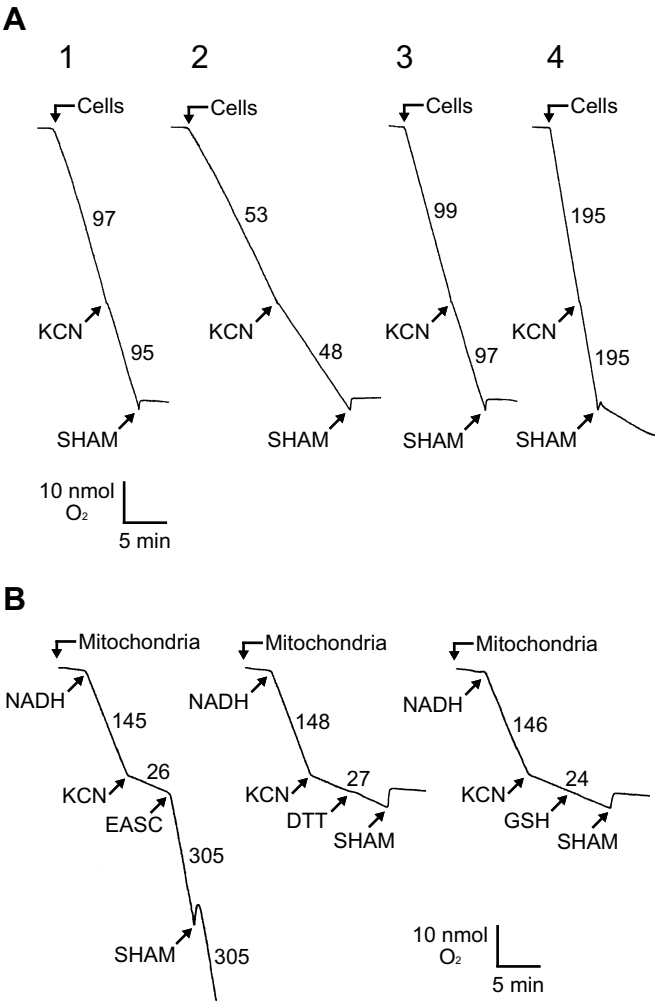

Fig. 3. AOX can be induced by antimycin A in the *alo1/alo1* mutant. (A) Measurement of respiratory activity of antimycin A-treated *C. albicans* cells. Trace 1, CAI4; trace 2, WH204; trace 3, WH205; trace 4, WH206. Rates on the respiration traces are in nmol O_2/h/mg wet cell. Respiratory inhibitors, 1 mM potassium cyanide (KCN) and 2 mM salicylhydroxamic acid (SHAM), were added as indicated by arrows. (B) Measurement of respiratory activity of mitochondria isolated from the CAI4 cells. Rates on the respiration traces are in nmol O_2/h/mg protein. To monitor the stimulatory effect on the respiratory activity, 1 mM EASC, 1 mM dithiothreitol (DTT) and 1 mM glutathione (GSH) were added as indicated by arrows.

about 12-fold increased by addition of EASC (Fig. 3B, trace 1). ASC showed the same stimulatory effect on the respiratory activity (data not shown). However, either dithiothreitol or glutathione could not stimulate the respiratory activity of *C. albicans* (Fig. 3B, trace 2, 3), suggesting that this stimulatory effect on the respiratory activity by EASC and ASC is not general to other physiological reductants. Notably, stimulation of the respiratory activity by EASC was still effective even after addition of salicylhydroxamic acid, a specific inhibitor of AOX [5], indicating that the stimulatory effect of EASC on the respiratory activity is not confined to the alternative respiratory pathway. Presumably, some components (e.g., cytochromes) common to both the conventional respiratory pathway and the alternative respiratory pathway may be involved in stimulation of the respiratory activity by EASC. The mechanism by which EASC stimulates the respiratory activity of *C. albicans* remains to be elucidated.

The present study shows that EASC activates cyanide-resistant respiration by inducing the expression of AOX in *C. albicans*. Although the precise mechanism by which EASC influences the expression of AOX remains unknown, the present study introduces a new aspect of regulation of cyanide-resistant respiration. The physicochemical properties and biological activities of EASC and ASC are very similar to each other [31]. As microorganisms contain EASC instead of ASC, it is reasonably presumed that in microorganisms EASC carries out various physiological functions similar to those of ASC in higher organisms. Our results showing activation of cyanide-resistant respiration by EASC in *C. albicans* raises the possibility that ASC may exert similar effects on cyanide-resistant respiration in higher plants. It will be of great interest to see if the expression of AOX can be induced by ASC in higher plants.

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