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Vacuolar acidification in *Saccharomyces cerevisiae* induced by elevated hydrostatic pressure is transient and is mediated by vacuolar H^+-ATPase

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Abstract We analyzed the vacuolar acidification in response to elevated hydrostatic pressure in *Saccharomyces cerevisiae*. The vacuolar pH, defined using 6-carboxyfluorescein, was directly measured in a hyperbaric chamber with a transparent window under high hydrostatic pressure. The vacuole of strain X2180 became acidified at the onset of pressurization to an extent dependent on the magnitude of pressure applied. A pressure of 40–60 MPa transiently reduced the vacuolar pH by about 0.33 within 4 min. The transient acidification was observed in the presence of D-glucose, D-fructose, or D-mannose as a carbon source, but not 3-*o*-methyl-D-glucose, ethanol, or glycerol, suggesting that the generation of CO_2 was involved in the process. A *vma3* mutant defective in vacuolar acidification showed no reduction of vacuolar pH when hydrostatic pressure was applied. This result indicates that the transient vacuolar acidification induced by elevated hydrostatic pressure is mediated through the function of the vacuolar H^+-ATPase.

Key words Hydrostatic pressure · *Saccharomyces cerevisiae* · Transient vacuolar acidification · Vacuolar H^+-ATPase · Chemical reaction of CO_2

Introduction

Living cells show a rapid response when they are exposed to disparate circumstances, e.g., high/low temperature, osmotic pressure, or extracellular pH. High hydrostatic pressure is a distinctive feature of the deep-sea environment, and this thermodynamic variable has a potentially disruptive influence on the biological activities of living organisms. In the yeast *Saccharomyces cerevisiae*, high hydrostatic pressure, above 100 MPa, induces cytoplasmic petite mutation (Rosin and Zimmerman 1977) and tetraploid or homozygous diploid forms (Hamada et al. 1992) or affects the ultrastructure of the cells (Kobori et al. 1995). A short duration of heat shock induces barotolerance allowing the cells to survive at 150 MPa (Iwahashi et al. 1991). However, few data have been reported concerning the physiological or biochemical responses of the cells to moderate or non-lethal hydrostatic pressures below 100 MPa.

The yeast vacuole is an acidic organelle, maintaining a low pH through the function of vacuolar H^+-ATPase (V-H^+-ATPase) on its membrane (Kakinuma et al. 1981). Acidification of vacuoles is essential for the activity of vacuolar enzymes, protein transport, and cytosolic ion homeostasis (Anraku et al. 1992a,b). We previously reported that hydrostatic pressure promoted the acidification of vacuoles in *S. cerevisiae*. Application of hydrostatic pressure for 1 h reduced the vacuolar pH by about 0.2 in both strains IFO2347 and X2180 (Abe and Horikoshi 1995). However, the precise kinetics of the pressure-induced acidification was unclear. In this study, we focused on the early time-course of vacuolar acidification in the primary response to elevated pressure, considering chemical reactions of CO_2.

Materials and methods

Yeast strains and culture conditions

A diploid formed by mating strains X2180-1A and X2180-1B was used in this study. Unless otherwise specified, cells were grown at 24°C in YPD broth (1% yeast extract, 2% bactopeptone, 2% D-glucose). To examine the effect of carbohydrates on vacuolar acidification, cells were grown in YP broth containing 2% of various carbohydrates. The

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vma3 mutant DV3T-A was kindly provided by Prof. Yasuhiro Anraku of Tokyo University (Tanida et al. 1995). The mutant and the parental strain YPH499 were cultured in YPD broth supplemented with 50 mM succinate/NaOH, pH 5.0 and 0.001% adenine sulfate.

Fluorescence analysis under high hydrostatic pressure

6-Carboxyfluorescein diacetate (6-CFDA), at a final concentration of 50 μM in YPD containing citric acid (pH 3.0), was used to label the yeast vacuoles with 6-carboxyfluorescein (6-CF) (Preston et al. 1989). Cells in the logarithmic phase of growth (2–3 × 10^7 cells/ml) were incubated with the dye for 1 h, washed twice in 5 mM MT buffer (5 mM 2-(*N*-morpholino) ethanesulfonic acid (Mes), 5 mM N-Tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid (Tes), pH 6.5), resuspended in MTG buffer (180 mM Mes, 180 mM Tes, 50 mM NaCl, 50 mM KCl, 100 mM D-glucose, pH 6.5) and put into a cuvette with a diameter of 6 mm. Unless otherwise specified, D-glucose was used as the carbon and energy source. After sealing it with parafilm, the cuvette was set on a hydrostatic chamber which had a transparent window with a diameter of 10 mm (Morita 1957). The labeled cells were analyzed using a CAF-110 fluorometer equipped with a FB-50 fiber irradiation outfit (Jasco, Tokyo, Japan). The point end of the fiber was connected to the transparent window. Vacuolar pH was determined as described by Preston et al. (1989). The fluorescence ratio was calculated and checked against *in vivo* calibration curves determined by equilibrating the vacuolar pH of 6-CF-labeled cells to that of the external pH in MTI buffer (180 mM Mes, 180 mM Tes, 50 mM NaCl, 50 mM KCl, 0.2 M ammonium acetate, 10 mM NaN_3, 10 mM 2-deoxyglucose, 50 μM carbonylcyanide *m*-chlorophenylhydrazone) at specified pH values under several hydrostatic pressures.

Determination of the amount of ethanol and cellular ATP

Cellular ATP was analyzed using an ATP analyzer (AF-110; Toadenpa-kogyo, Tokyo, Japan). The CO_2 generation was estimated by measuring the ethanol production, which was enzymatically determined using a kit produced by Boehringer (Mannheim, Germany) to determine the ethanol concentration in foodstuffs and other materials, following the changes in [NADH] at 340 nm.

Results and discussion

Figure 1 shows *in vivo* calibration curves obtained under several hydrostatic pressures. Only a slight increase in the fluorescence ratio was observed within the pH range of 5.5–7.0 in MTI buffer as the hydrostatic pressure was elevated. Therefore, we conclude that hydrostatic pressure has a negligible effect on the fluorescence ratio as determined using 6-CF.

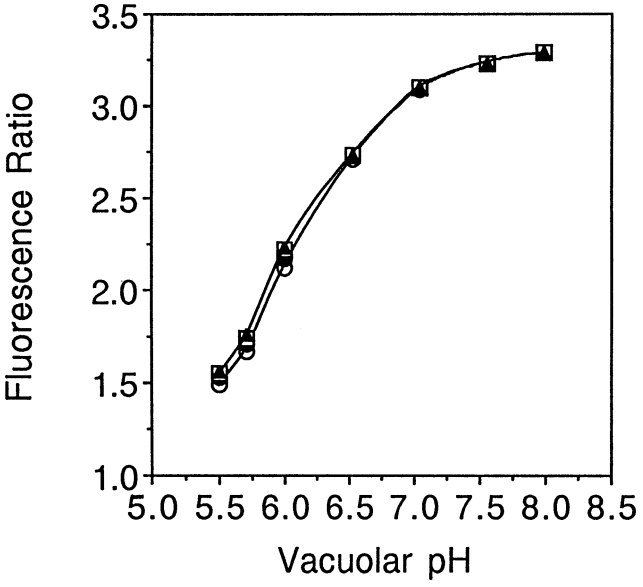

Fig. 1. *In vivo* calibration of vacuolar pH under hydrostatic pressure. The fluorescence ratio of 6-carboxyfluorescein- (6-CF) labeled cells in MTI buffer at several specified pH values was determined at atmospheric pressure (*open circles*); 20 MPa (*closed circles*); 40 MPa (*squares*); and 60 MPa (*triangles*)

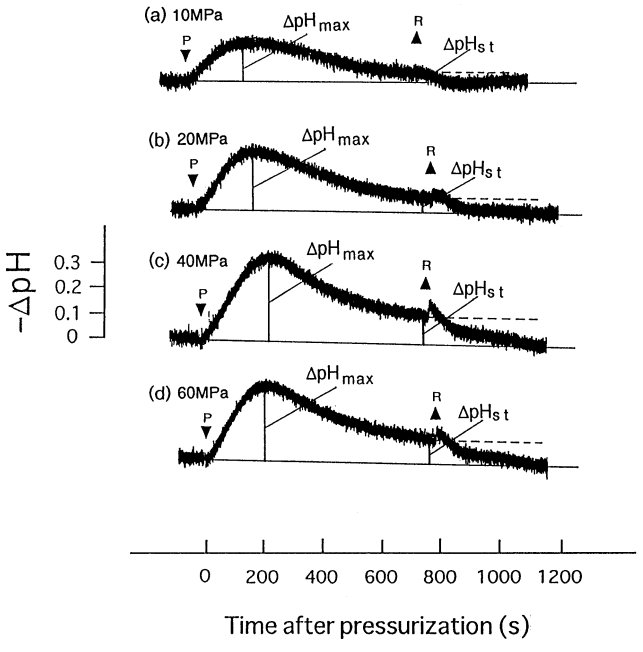

Fig. 2. Time course of vacuolar acidification after the application of hydrostatic pressure. Cells of strain X2180 were subjected to hydrostatic pressures of 10 MPa (**a**), 20 MPa (**b**), 40 MPa (**c**), and 60 MPa (**d**) at *P*, and pressure was subsequently removed at *R*. ΔpH_{max}, maximal change in vacuolar acidification; ΔpH_{st}, steady level of acidification

At atmospheric pressure, the vacuolar pH of strain X2180 was 6.30 (SD ± 0.039). Figure 2 shows a series of typical recorded changes in vacuolar pH (ΔpH = vacuolar pH at high hydrostatic pressure – vacuolar pH at atmo-

Table 1. Changes in vacuolar pH induced by hydrostatic pressure

Pressure (MPa)	Changes in vacuolar pH			
	ΔpH_{max} ± SD	(*n*)	ΔpH_{st} ± SD	(*n*)
5	0.070 ± 0.017	(3)	0.030 ± 0.020	(3)
10	0.113 ± 0.006	(3)	0.057 ± 0.006	(3)
20	0.227 ± 0.017	(7)	0.120 ± 0.030	(3)
30	0.285 ± 0.021	(3)	0.165 ± 0.007	(3)
40	0.326 ± 0.014	(11)	0.206 ± 0.018	(5)
60	0.324 ± 0.019	(7)	0.220 ± 0.026	(3)

All data are represented as mean changes (±SD) under each hydrostatic pressure with respect to the initial measurements at atmospheric pressure. ΔpH_{max}, maximal change in vacuolar acidification; ΔpH_{st}, steady level of acidification.

Table 2. Changes in vacuolar pH induced by a pressure of 40 MPa in MTG buffers at specified pH values

External pH	Changes in vacuolar pH (at 40 MPa)	
	ΔpH_{max} ± SD	(*n*)
5.5	0.323 ± 0.042	(3)
6.5	0.326 ± 0.014	(11)
7.0	0.287 ± 0.023	(3)
7.5	0.297 ± 0.029	(3)

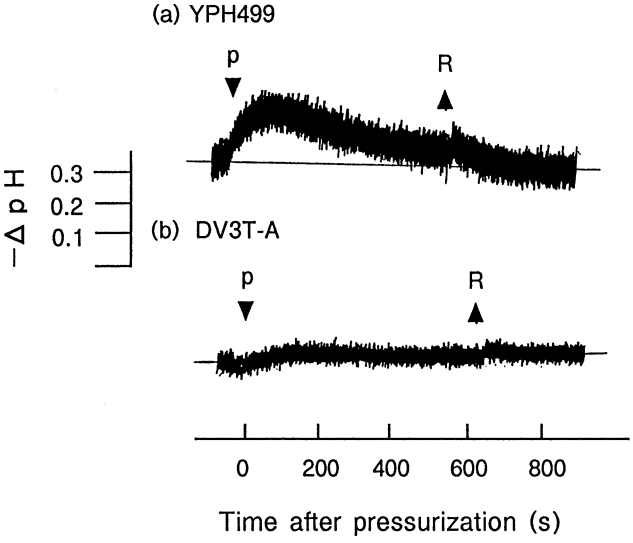

Fig. 3. Time course of vacuolar acidification in the vacuolar mutant after the application of hydrostatic pressure. Cells were subjected to a pressure of 40 MPa at *P*, and pressure was subsequently removed at *R*. **a** parental strain YPH499, **b** *vma3* mutant DV3T-A

spheric pressure). The yeast vacuole was transiently acidified to an extent dependent on the magnitude of pressure applied (Fig. 2a–d). There was an immediate sharp decrease in pH after the application of hydrostatic pressure. The ΔpH rose to a maximum (ΔpH_{max}) within 200 s and then gradually fell to a steady level (ΔpH_{st}). Subsequently after decompression, the vacuolar pH gradually returned to the original level observed at atmospheric pressure. No substantial change in the external pH (<0.02) of the buffer was observed during the analysis. The pressure-induced acidification was reversible after the initial pressure treatment (data not shown). Overall values of the transient acidification induced by hydrostatic pressure are shown in Table 1. Interestingly, the ΔpH_{st} values closely corresponded to those of our previous report (Abe and Horikoshi 1995), even though the experimental conditions differed. The ΔpH_{max} values differed only slightly under acidic, neutral, or alkaline conditions; thus, we conclude that the transient acidification was mostly an intracellular event, not due to passive diffusion of protons from external buffers (Table 2).

The VMA3 gene product is subunit c of the V-H^+-ATPase which constitutes a proton pore in the vacuolar membrane, and *vma3* mutants completely lack V-H^+-ATPase activity (Umemoto et al. 1990, Tanida et al. 1995). Consequently, the *vma3* mutant DV3T-A had near-neutral vacuoles (vacuolar pH = 6.76 ± 0.03, $n = 4$). As a result, DV3T-A cells failed to show the transient acidification when hydrostatic pressure was applied (Fig. 3). Bafilomycin A_1, a specific inhibitor of V-H^+-ATPase, caused a rapid and significant alkalization of vacuoles at both atmospheric pressure and high hydrostatic pressure (data not shown). Therefore, we conclude that the pressure-induced acidification was mediated by the V-H^+-ATPase, and was not due to simple changes in the equilibrium of the vacuolar medium. It is still unclear whether V-H^+-ATPase is directly activated by elevated hydrostatic pressure, in contrast to Na^+, K^+-ATPase (Chong et al. 1985) or Ca^{2+}-ATPase (Heremans and Wuytack 1980) which are sensitive to inactivation by elevated pressure.

To estimate the CO_2 generation in a short time-frame under hydrostatic pressure, we examined ethanol production in the presence of D-glucose. Ethanol production at 40 MPa was 75% of that produced at atmospheric pressure (Fig. 4). This means that a considerable amount of CO_2 was generated under hydrostatic pressure. Mean-while, no significant change was observed in cellular ATP levels during the incubations at both pressures. The transient vacuolar acidification was also caused in the presence of D-fructose or D-mannose when hydrostatic pressure was applied. In contrast to the results with fermentable sugars, hydrostatic pressure did not affect the vacuolar pH in the presence of ethanol or glycerol, even though cellular ATP levels remained normal, similar to ATP levels causing acidification in D-glucose. In contrast, 2-deoxy-D-glucose, which had the effect of depleting ATP, inhibited the pressure-induced acidification of vacuoles (data not shown).

Here, we consider the pressure-induced acidification from the following two view-points: (1) chemical reactions of CO_2 in solution, and (2) cytoplasmic pH homeostasis. The gas CO_2 is quite soluble in water; more than 99% of aqueous CO_2 exists as the dissolved gas and less than 1% as carbonic acid, H_2CO_3, which partly dissociates to give H^+, HCO_3^-, and CO_3^{2-} at atmospheric pressure. The reaction

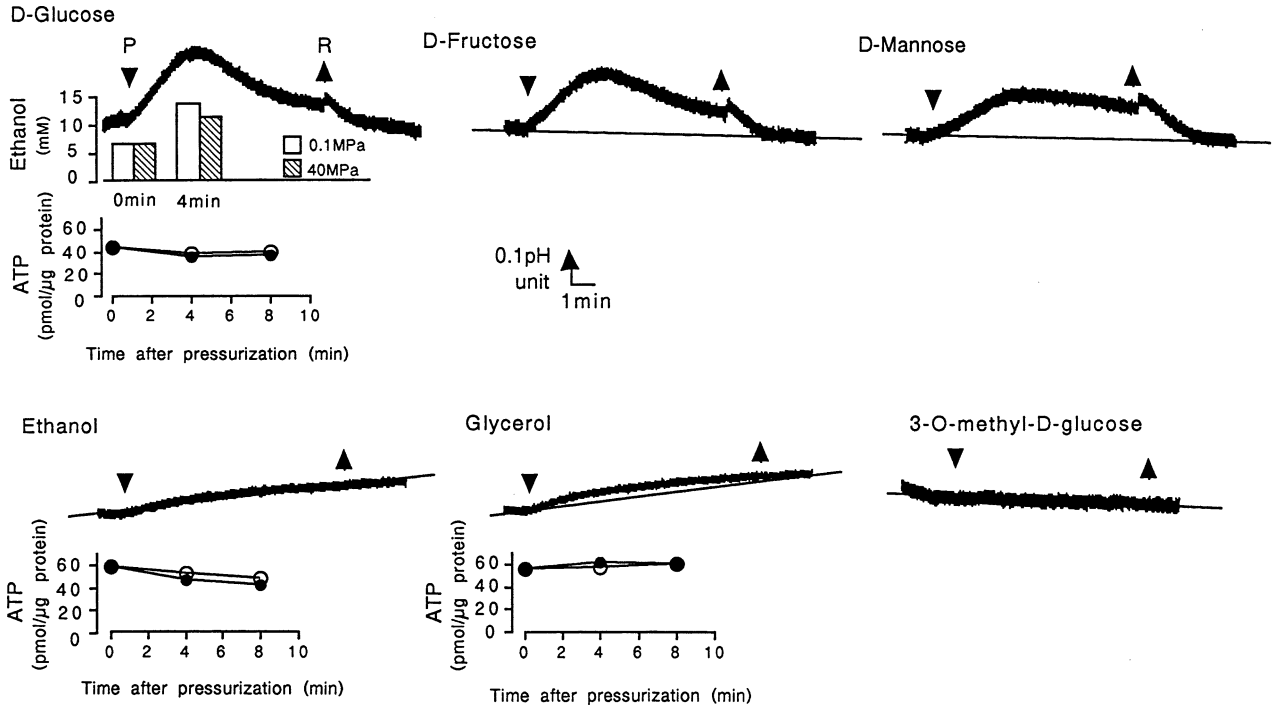

Fig. 4. Time course of vacuolar acidification, concentration of ethanol, and the amount of cellular ATP, in the presence of various carbohydrates (100 mM) after the application of hydrostatic pressure. Cells were subjected to a pressure of 40 MPa at *P*; pressure was subsequently removed at *R*. *Open circles*, cellular ATP at atmospheric pressure; *closed circles*, cellular ATP at 40 MPa

volume (ΔV) of the reaction $CO_2 + H_2O \rightarrow H^+ + HCO_3^-$ is negative (–26.0 ml/mol), which means that the dissociation of the weak acid is markedly facilitated by elevated pressure. Therefore, large numbers of protons generated by the dissociation of carbonic acid could be accumulated in the cytoplasm under high hydrostatic pressure.

Glycolytic intermediates such as glucose-6-phosphate and fructose-6-phosphate might also release protons with elevated pressure, since the reaction volumes of the weak acids are negative. It has yet to be determined whether cytoplasmic phosphate ions function as a powerful buffer, since the reaction $H_2PO_4^- \rightarrow HPO_4^{2-} + H^+$ could be facilitated by elevated hydrostatic pressure. It would be worthwhile in future studies to measure the cytoplasmic pH in the presence or absence of fermentable sugars under hydrostatic pressure. Yeast vacuoles contain high concentrations of amino acids such as arginine, lysine, and histidine; the increased numbers of cytoplasmic protons might be taken up into the vacuoles and neutralized by these amines at high hydrostatic pressure. The contribution of amines in pH regulation should be examined by analyzing the pressure-induced acidification in strains with varying amounts of amines in the vacuoles. It is also likely that proton efflux from the vacuoles via the Ca^{2+}/H^+ antiport, amino acids/H^+ antiport, or passive diffusion is slowed by elevated pressure, which would lead to an increase of protons in the vacuoles.

It has been postulated that the yeast vacuole plays an important role in cytosolic ion homeostasis (Anraku et al. 1992a,b). In hepatocytes, the V-H^+-ATPase makes an important contribution to the regulation of cytosolic pH (Wadsworth and van Rossum 1994). To maintain a favorable cytoplasmic pH, the yeast vacuole may serve as a proton sequestrant when hydrostatic pressure is applied. These findings make it clear that the chemical reactions of low molecular weight compounds should be noted to elucidate the effect of hydrostatic pressure on living organisms.

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