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MiniReview

The heat shock and ethanol stress responses of yeast exhibit extensive similarity and functional overlap

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Abstract

Sublethal heat and ethanol exposure induce essentially identical stress responses in yeast. These responses are characterized by the induction of heat shock proteins, proteins requiring a temperature above about 35°C or ethanol levels above a threshold level of 4–6% (v/v) for strong induction. One induced protein, Hsp104, contributes to both thermotolerance and ethanol tolerance, while others are anti-oxidant enzymes. Heat and ethanol stress cause similar changes to plasma membrane protein composition, reducing the levels of plasma membrane H^+-ATPase protein and inducing the plasma membrane-associated Hsp30. Both stresses also stimulate the activity of the fraction of H^+-ATPase remaining in the plasma membrane. The resulting enhancement to *catalysed* proton efflux from the cell represents a considerable energy demand, yet may help to counteract the adverse effects for homeostasis of the increased membrane permeability that results from stress.

Keywords: *Saccharomyces cerevisiae*; Stress responses; Stress tolerance; Ethanol; Heat shock proteins; Plasma membrane H^+-ATPase

1. Introduction

The high ethanol tolerance of *Saccharomyces cerevisiae* and the high ethanol yields of *S. cerevisiae* fermentations are among the more commonly known attributes of this organism and a major factor contributing to the widespread attention this yeast has received. Although many different yeasts are usually present at the beginning of wine fermentations, *S. cerevisiae* soon outgrows the others as ethanol levels rise (see [1] for literature). However, even this yeast has a finite ethanol tolerance, the rising ethanol level during batch fermentation on high concentrations of sugar substrates acting initially to reduce growth and fermentation rates and, at growth-inhibitory levels, to adversely affect cell viability (see [2] for early literature). The minimal concentration of ethanol that is totally inhibitory for *S. cerevisiae* growth is strongly influenced by medium composition and is remarkably strain-dependent [1]. There is an extensive literature on the alterations to the phospholipid and sterol composition of yeast cell membranes with adaption to growth in the presence of ethanol (reviewed in [1,3,4]).

This review summarises the evidence that many major changes induced in yeast by stressful ethanol levels are identical to those caused by heat stress; that a synergy exists between heat-induced and ethanol-induced damages, and that this synergy results in the adverse influences of ethanol being more severe at higher temperatures. Ethanol toxicity is

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generally attributed to the preferential partitioning of ethanol to the hydrophobic environment of lipid bilayers, resulting in a disruption of membrane structure that adversely affects many membrane-associated processes [1–6]. However, both heat shock and ethanol exposure will cause, in addition to membrane disordering, increases in protein denaturation. Since the membrane-disruptive effects and membrane-lipid changes associated with ethanol exposure have been reviewed quite recently [1,3–6], this account will focus primarily on the proteins of heat and ethanol stress protection and the very limited evidence of the signals that may lead to their induction.

Increased stress tolerance is generally inducible. In vegetative yeast cells, ethanol tolerance is frequently induced under the same conditions as those that lead to increased thermotolerance [9]. However, whereas a pre-exposure to heat shock leads to acquisition of ethanol tolerance, the converse is not the case. Ethanol pre-exposure actually hypersensitizes cells to heat [2,15]. A great many factors determine tolerances of yeast to heat or ethanol. Besides heat shock proteins (Section 2.1 and Section 2.2), membrane lipids (Section 4.1), plasma membrane H^+-ATPase action (Section 4.2), trehalose (Section 4.4), and diverse other changes resulting from heat or ethanol exposure may all be important (see [9] for review of factors influencing thermotolerance).

2. Gene induction due to sublethal heat shock or ethanol exposure

2.1. Induction of heat shock proteins

Sublethal heat shock and ethanol stress induce practically identical changes to protein synthesis. Above a critical threshold level, both stresses strongly induce heat shock proteins, with simultaneous suppression of synthesis of most proteins made prior to the stress [7–11]. Heat shock proteins are evolutionarily conserved proteins, originally characterized on the basis of their strong induction by heat shock. They are also induced by a number of chemical agents including ethanol. The *S. cerevisiae* genes induced by ethanol seem to be mostly identical to those induced by heat shock. For example, the ethanol induction of Hsp70 in *S. cerevisiae* is due to expression of the same three Hsp70 genes that display a strong heat induction [11].

The threshold concentration for ethanol to cause appreciable heat shock protein induction in vegetative yeast cultures growing at 25°C is between 4% and 6% (v/v). Ethanol additions below this level produce little alteration to the pattern of protein synthesis [11]. Individual heat shock proteins are optimally induced at different levels of ethanol above 4–6%. The induction of Hsp104 (important for ethanol and heat tolerance [7]), Hsp70 and Hsp26 increases progressively as the ethanol addition is increased from 4% to 10%, in contrast to Hsp82 and the plasma membrane Hsp30, both of which are maximally induced at 6% ethanol [11]. This observation that different ethanol levels are required for maximal induction of different heat shock proteins suggests that the systems for expression of heat shock genes can sense different sublethal ethanol concentrations. Only for Hsp104, and to some extent Hsp70, has yeast molecular genetics provided clear evidence of the protein making a direct contribution to thermotolerance and ethanol tolerance. Loss of Hsp104 protein has been shown to reduce both ethanol tolerance, tolerance to heat, and heat induced tolerance to ethanol (see [8] for literature).

2.2. Induction of anti-oxidant proteins

About 1% of electrons are directly transferred to oxygen in mitochondria under normal oxygen pressures, mainly by leakage at the cytochrome bc_1 complex (see [12,13] for literature). This incomplete reduction of molecular oxygen by the respiratory chain of the mitochondrion is an important source of endogenous superoxide anion production in aerobic cells. Production of such reactive oxygen species (ROS) increases dramatically under conditions of pure oxygen, ethanol or heat stress and these ROS, if not neutralized, cause severe oxidative damage to proteins, lipids and to DNA [12,13]. It has also been suggested that the increased portioning of molecular oxygen from the aqueous to the lipid phases of cells at higher temperatures may contribute to enhanced membrane lipid oxidation [14].

Certain major anti-oxidant activities are increased by heat stress to counteract this increased ROS production [12,13]. ROS are neutralized in yeast by both

non-enzymatic and enzymatic processes. The non-enzymatic anti-oxidant defence is primarily the glutathione pool. Of the enzymatic defences, the enzymes catalase and superoxide dismutase, catalysing degradation of hydrogen peroxide and superoxide radicals, respectively, are particularly important. Although heat shock and 8% ethanol do not alter levels of either glutathione or the cytoplasmic CuZn superoxide dismutase [12,13], both stresses increase the activities of cytoplasmic catalase T (the product of the *CTT1* gene) and the mitochondrial manganese form of superoxide dismutase (MnSOD, the product of the nuclear *SOD2* gene) [12–17]. Loss of *CTT1* slightly reduces thermotolerance, except in cells of high cAMP-dependent protein kinase activity, reflecting the tendency for increased ROS production in respiratory cultures at higher temperatures [16,17]. The importance of MnSOD elevation by heat and ethanol, and therefore of efficient trapping of superoxide radicals within mitochondria, is shown by the high sensitivity of *sod2* mutants to the lethal effects of ethanol [13]. The damage ROS can inflict is also reflected in the increased ethanol tolerance of respiratory-deficient petites, cells that have lost ROS production by the respiratory chain [13,15].

There are a number of reports of the induction of a cytochrome P_{450} in yeast by ethanol [3]. It is still not clear whether this reflects significant removal of ethanol from yeast cells by an endoplasmic reticulum-associated ethanol-oxidizing activity.

2.3. Gene promoter elements responsive to heat and ethanol

Two gene promoter elements, the heat shock element (HSE) and the general stress responsive element (STRE), have been shown to direct activation of yeast heat shock genes with heat stress. The HSE consists of alternating repeats of the sequence nGAAn at each half-turn of the DNA helix [18,19], while the STRE consensus sequence is AGGGG or CCCCT [17,20]. These two elements differ in the diversity of stresses that cause their activation. The HSE is primarily activated only by heat shock in vegetative cells, losing its heat-inducibility at stationary phase [19]. While we could demonstrate activation of this element in a HSE-*lacZ* reporter gene fusion by methanol [21], we obtained very little activation of the same sequence by ethanol [19]. This is surprising since ethanol is a good inducer of heat shock proteins [10,11]. Possibly a particular sequence context of the nGAAn repeats, absent in the HSE-*lacZ* reporter construct that we used, is needed for efficient HSE induction by ethanol. Ethanol induction of the STRE is readily demonstrable, as also is STRE activation by several other stresses, including heat shock and osmo-stress [17,20]. Another major difference between the HSE and the STRE is that the latter element is under negative regulation by cAMP-dependent protein kinase, while HSE activity is unaffected by the activity of this kinase [17]. Some heat-inducible genes of *S. cerevisiae* are controlled by both elements, a few (e.g. *SSA4*) have the HSE alone, while a third category (e.g. *CTT1*, *SOD2* and the genes of trehalose biosynthesis) appear to have only the STRE [20]. It is conceivable that ethanol may partly induce STRE activity through the oxidative stress that it causes (Section 2.2), since oxidative stress is now known to influence the activity of certain STRE-regulated promoters [13].

3. Common damages of heat stress and ethanol stress

3.1. Adverse effects on membrane-associated processes

Both heat stress and ethanol increase the permeability of membranes, thereby adversely affecting membrane-associated processes. The necessity of membrane integrity for chemi-osmotic coupling of electron transport to ATP synthesis causes synthesis of ATP from respiration to be considerably more sensitive to inhibition by ethanol than ATP generation by glucose fermentation. Growth of *S. cerevisiae* at high temperatures or in the presence of ethanol causes an enhanced induction of respiratory-deficient petites [2,22]. Since petites have mitochondria possessing a non-functional respiratory chain, this petite induction may reflect adverse effects of these stresses on respiratory chain assembly. However, another important factor is probably the beneficial effect of losing ROS production by the respiratory chain since, as mentioned in Section 2.2, respiratory deficiency confers an enhanced ethanol

tolerance.

Plasma membrane-associated events are especially susceptible to stress. Ethanol has been shown to inhibit glucose, maltose, ammonium and amino acid uptake, as well as to cause leakage of potassium, amino acids and nucleotides (see [3] for literature). At the plasma membrane, the increased passive proton influx due to stress will act to dissipate the electrochemical potential gradient maintained across this membrane by the action of plasma membrane H^+-ATPase. This adversely affects those vital functions for which a plasma membrane electrochemical gradient is essential, processes such as active uptake of amino acids and ammonium ions, the maintenance of potassium balance, and the regulation of intracellular pH [23]. Decreased proton motive force at the plasma membrane, or intracellular pH decline, have been observed both with ethanol addition [3,24] and with heat shock [25]. Intracellular pH decline may be a major factor contributing to the inhibition of fermentation rate, reflecting a decreased glycolytic flux, observed in cells subjected to heat stress [26] or ethanol exposure [3].

3.2. Synergy between effects of heat and ethanol

With so many changes induced by heat being the same as those induced by ethanol, it is not surprising that ethanol acts in a synergistic way to increase the damage caused by heat [2,22,27]. The stress-sensing systems of yeast appear to be at least partly responsive to this synergy, since ethanol lowers by 3°C the temperature for maximal induction by heat of the heat shock response, as is evident from β-galactosidase production from a HSE-*lacZ* gene [28]. The adverse effects of ethanol are rendered considerably more severe by increasing the temperature, the presence of ethanol levels above 3% causing the optimal and maximal temperatures of growth to become appreciably depressed and thermal death on exposure to extreme temperatures to be considerably enhanced [2,22,27]. That both of these effects result from a non-specific, lipid-disordering interaction between ethanol and the cell membrane is suggested by studies using alkanols of increasing aliphatic chain length. The effects of these alkanols are directly related to their lipid solubility [2,6]. Methanol is the least toxic, inactivating cells only above 18–20%, even though a HSE–*lacZ* fusion is maximally induced at about 10% methanol [21]. Ethanol is cytotoxic at lower levels [2], inducing most Hsps maximally at 4–10% [10,11]. Similarly, whereas 6.0% methanol is required to decrease optimal temperature for heat induction of the heat shock response by 3°C, only 2.6% ethanol is needed to achieve the same effect [28]. As alkanol chain length and hydrophobicity increase, progressively smaller amounts of the alkanol are needed to either inhibit yeast cell growth [2,6], to achieve the 3°C depression in optimal temperature for heat activation of the HSE sequence [28], or induce heat shock proteins in the absence of heat [29]. That more hydrophobic alkanols are better chemical inducers of heat shock proteins may reflect their increased capacity to destabilize the hydrophobic interactions that maintain protein conformations (Section 2.3).

4. Plasma membrane lipid changes, plasma membrane activities, and the protective effects of trehalose may all contribute to adaptation to heat and ethanol stress

4.1. Changes to lipid composition

Evidence is steadily accumulating that growth at high temperatures and growth in the presence of ethanol are correlated with decreased unsaturation of the lipids of yeast cell membranes [1,3,4,14,30]. Ethanol also causes a reduced and altered membrane sterol content [3,30]. These responses can be relatively rapid [30] and appear to be an adaptive response to counteract the less ordered, more 'fluid', state of membranes with higher temperatures or ethanol exposure. There is now an extensive literature on how different lipid supplementations to the growth medium of yeasts correlate with effects on ethanol toxicity and ethanol productivities (reviewed in [3]). However, while these data are reasonably convincing, there is as yet no *direct* proof that lipid changes can *alone* directly alter stress tolerances.

4.2. Changes to plasma membrane H^+-ATPase level and activity

Plasma membrane H^+-ATPase is the major activity responsible for maintaining the electrochemical

potential gradient across the plasma membrane [23]. When yeast cells are exposed to sublethal heat shock [25,31] or sublethal ethanol levels [24,30,32], the activity of this H^+-ATPase is dramatically stimulated. This activation of the H^+-ATPase by heat or ethanol occurs in vivo, being readily detectable as an increased proton extrusion by intact cells [25,31], and it is not lost during purification of membranes for ATPase assay [24,30,32]. It reflects a stress-induced modification of the ATPase, the mere addition of ethanol to the in vitro ATPase assay reaction leading not to activation of the ATPase but instead to a partial inhibition [30,32]. Remarkably, this ATPase inhibition due to ethanol addition to the in vitro assay is less if the membranes are from ethanol-adapted cells [30], possibly due to the plasma membrane lipid changes that occur with adaptation to ethanol (Section 4.1).

Although heat shock and ethanol exposure both stimulate plasma membrane H^+-ATPase, *protein* levels of this proton-pumping ATPase actually show a rapid initial decline with short periods of either stress [11,33], stabilizing at 35–50% of their initial level 20–30 min into the stress period (our unpublished results). This rapid depletion of plasma membrane H^+-ATPase protein contrasts with the stimulation of the residual H^+-ATPase remaining in the membrane 20–30 min into the period of stress [30–32]. Reduction in H^+-ATPase *protein* levels and stimulation of that fraction of the H^+-ATPase that remains in the membrane therefore both occur simultaneously when cells are suddenly exposed to heat or ethanol stress. Both processes probably influence the capacity of the cells to reestablish and sustain homeostasis immediately after imposition of stress. The increased plasma membrane H^+-ATPase activity in heat- or ethanol-stressed cells will cause an enhanced *catalysed* proton efflux. Subject to certain preconditions, this proton extrusion will counteract the dissipation of proton motive force resulting from stress-induced increases in membrane permeability. This is probably one reason that mutations that alter plasma membrane H^+-ATPase activity can influence cellular tolerances of both ethanol and heat [31,33]. Imposition of heat or ethanol stress demands a dramatically increased expenditure of energy as cells struggle to re-establish and subsequently maintain homeostasis. Diverse other factors that affect homeostasis, in addition to H^+-ATPase activity, will influence cellular tolerances to heat and to ethanol. For example, cells survive heat stress best at pH 6–7 [34], when extracellular pH approximates to intracellular pH. The same probably applies to exposure to alkanols and other agents that increase membrane permeability.

4.3. Induction of Hsp30

Several stresses lead to induction of Hsp30, a highly hydrophobic integral membrane protein and the only heat shock protein tightly associated with the yeast plasma membrane [11,33]. Hsp30 is induced to similar levels by heat shock and by treatment with 6% ethanol, although induction appears to be appreciably less with 8% ethanol [11]. We have recently shown that Hsp30 is a stress-inducible negative regulator of plasma membrane H^+-ATPase activity (manuscript in preparation). Cells lacking Hsp30 give lower final biomass yields in batch fermentations. In addition, they take longer to adapt to enable subsequent growth when exposed to non-growth-inhibitory levels of several energy-demanding stresses, including low pH, osmo-stress, the presence of weak organic acids and 10% ethanol. Despite this, Hsp30 loss does not affect thermotolerance or ethanol tolerance levels.

Plasma membrane H^+-ATPase action is thought to consume 15–50% of all ATP generated by unstressed cells [23]. The greater ATP consumption by this H^+-ATPase on imposition of a heat or ethanol stress will represent a major energy demand on the cell. Once adapted to the stress so that growth can resume, *hsp30* mutant cells subsequently maintain lower ATP levels, consistent with their lack of Hsp30 down-regulation of H^+-ATPase activity (manuscript in preparation). It is possible, therefore, that the function of Hsp30 may be primarily for energy conservation, down-regulating the stress-activation of the plasma membrane H^+-ATPase so that this ATPase does not totally deplete the energy reserves of the cell.

4.4. Membrane-protective effects of trehalose induction

Mansure et al. [34] recently noted a positive correlation between trehalose levels of yeast strains and

their ability to withstand 10% ethanol. They also obtained evidence that trehalose reverses electrolyte leakage from ethanol-treated yeast cells and carboxyfluorescein leakage from liposomes. The strong trehalose induction in cells subjected to heat shock or ethanol [9] may therefore partially counteract the deleterious effects of the increased membrane permeabilization associated with these stresses. Trehalose may also help stabilize proteins, since it stabilizes proteins against thermal denaturation in vitro [35].

5. The cell membrane in the sensing of stress

A major trigger for heat shock protein induction is thought to be the cytoplasmic accumulation of aberrant or partially denatured protein [7–9,18]. Ethanol may be an inducer through its destabilization of the hydrophobic interactions within native protein structures. This may expose localized hydrophobic regions on proteins, leading to the association of chaperones (notably Hsp70) with these destabilized proteins. The concomitant depletion of the 'free' chaperone pool within cells may be the signal for activation of heat shock genes (reviewed in [7,9,18]).

In yeast, there also exists indirect evidence for the cell membrane being involved in the sensing of heat stress. Depolarization of this membrane, as with plasma membrane H^+-ATPase inhibition, rapidly renders cells incapable of a heat shock response even though they still retain the capacity for efficient protein synthesis [36]. In addition, the lipid composition of the plasma membrane affects stress signalling. Altering the saturation of plasma membrane phospholipids in cells carrying a HSE-*lacZ* reporter plasmid dramatically affects the temperature range over which this HSE–*lacZ* fusion is activated by heat (B. Curran; unpublished results). Whether STRE promoter element activity (Section 2.3) or the response to ethanol are also influenced by membrane lipid changes has yet to be established, but it seems probable that an effect will be noted. The proton motive force at the plasma membrane or the fluidity of this membrane are both potentially very sensitive monitors of environmental change. This may make the plasma membrane the ideal location for stress-sensors. It provides the location for Sln1p, a transmembrane osmo-stress-sensing histidine kinase [37], and phospholipase C, a crucial enzyme for phosphoinositide-linked signalling. Stimulation of phospholipase C appears to be responsible for many of the longer-term changes induced by ethanol in mammalian systems [38], although there is as yet no evidence that stimulation of phospholipase C influences ethanol adaptation in yeast.

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