Diethyl phthalate increases the percentage of unsaturated fatty acids with a concomitant decrease in cellular heat shock sensitivity in the yeast *Saccharomyces cerevisiae*

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In the past it has been reproducibly demonstrated that 37 °C-grown DBY747 yeast cells have 29% more unsaturated fatty acids and a 3 °C higher maximal heat shock response (HSR) than their 25 °C counterparts. Suddenly the HSR and lipid profiles of cells grown at 25 °C and 37 °C became indistinguishable from one another. This paper reports an aberrantly high level of unsaturated fatty acids and an abnormally insensitive HSR in cells grown at 25 °C in yeast nitrogen base (YNB) that has been reconstituted from dehydrated medium packaged in 'new' plastic containers. Effective even at a 1:600 dilution of reconstituted medium in laboratory-made YNB, the 'active ingredient' was identified using a combination of HPLC and mass spectroscopy as diethyl phthalate (a plasticising agent). Furthermore, the same levels of increase in the percentage of unsaturated fatty acids and decrease in the sensitivity of HSR were found in cells grown in laboratory-made YNB that contained as little as 36 µM pure diethyl phthalate. This compound nevertheless failed to elicit an observable effect on cellular growth rate at levels up to and including 144 µM. These results suggest that diethyl phthalate causes yeast cells to accumulate high levels of unsaturated fatty acids with a concomitant decrease in the sensitivity of the HSR, without compromising overall cellular function. They also support earlier work that suggested that the HSR is exquisitely sensitive to the level of unsaturated fatty acids present in yeast cells.

Keywords: Diethyl phthalate, lipids, yeast, heat shock response

INTRODUCTION

In common with other living organisms, normal metabolic functions are suppressed and genes encoding heat shock proteins induced when yeast cells are exposed to a sublethal heat shock (reviewed by Mager & De Kruijff, 1995). The accumulation of denatured cellular proteins is widely regarded as the trigger for the induction of these genes but the precise mechanism by which heat stress is transduced into an intracellular signal remains largely unknown (reviewed by Parsell & Lindquist, 1993). The heat shock response (HSR) is transient, with a rapid induction of heat shock proteins when yeast cells are shifted from 25 °C to 37 °C, followed by a decline to a new basal level of activity, which remains constant for

as long as the cells are maintained at the higher temperature (Miller *et al.*, 1979; Slater & Craig, 1987; Sorger, 1990, 1991). This observation is not consistent with a HSR model in which thermally denatured proteins are the trigger for the response because a continuous heat shock should give rise to continual protein denaturation, thus necessitating the need for continual activation of the heat shock genes. Instead, the transient nature of the HSR strongly suggests that the heat-stress transduction mechanism can become desensitised, depending upon the prevailing conditions in the cell.

Intriguingly, the plasma membrane has been implicated in mechanisms of cellular stress (reviewed by Vigh *et al.*, 1998), and membrane fluidity varies with changes in environmental temperature in a wide variety of different organisms, including *Saccharomyces cerevisiae* (Hunter

Abbreviations: HSE, heat shock element; HSR, heat shock response.

& Rose, 1972; Cronan, 1978; Okuyama *et al.*, 1979; Suutari *et al.*, 1990). Coote *et al.* (1991) noted that the yeast plasma membrane becomes leaky as a consequence of heat stress, resulting in an increased permeability to extracellular protons. They suggested that this intracellular acidification acted as a trigger for inducing tolerance to an otherwise lethal temperature challenge. Furthermore, Coote *et al.* (1994) used an ATPase mutant of *Saccharomyces cerevisiae* to demonstrate that this membrane-bound proton pump plays a key role in cellular thermotolerance. The activity of this enzyme has also been shown to affect both the level of expression of heat shock genes and the duration of time over which they are induced (Panaretou & Piper, 1990) whereas in a separate study, minor intracellular acidification arising from ionophore treatment has been correlated with the expression of HSP70 in yeast (Weitzel *et al.*, 1987). Furthermore, Kamada *et al.* (1995) provided evidence that thermal stress triggers the expression of the yeast *PKC1* gene, by stretching the plasma membrane. Thus a number of disparate observations provide support for the view that cellular stress sensitivity is intimately associated with membrane structure and function. The direct manipulation of cellular fatty acids provides further evidence for this (Carratu *et al.*, 1996; Chatterjee *et al.*, 1997).

Carratu *et al.* (1996) found a decrease in the cellular heat shock sensitivity and an increase in unsaturated fatty acids in yeast cells overexpressing the *ole1* gene product. Previously published work from this laboratory (Chatterjee *et al.*, 1997) demonstrated an equally strong correlation between the heat shock sensitivity of yeast and the type of fatty acid present in the cells. Expression of β -galactosidase from a heat shock reporter gene revealed that the temperature of maximum induction was 45 °C, 47 °C or 49 °C, depending on the type and percentage of unsaturated fatty acids present in cells after they had been grown on different lipid sources under anaerobic conditions. We also demonstrated that cells grown at 37 °C have 29% more unsaturated fatty acids and a 3 °C higher maximal HSR than cells grown at 25 °C; an alteration in lipid profile and HSR sensitivity that we propose provides a possible mechanism to explain the transient nature of the HSR.

Despite multiple reproducible repeats of experiments that revealed these differences, suddenly the profiles of β -galactosidase induction from cells grown at 25 °C and 37 °C became indistinguishable from one another. Even more intriguingly, for no apparent reason the lipid profiles from the two sets of cells also became the same. Here, after many months of exhaustive analysis we provide an explanation for this abrupt change; an explanation that adds even further weight to the hypothesis that the HSR is intimately associated with membrane structure.

METHODS

Strains. *Saccharomyces cerevisiae* strain DBY747-HSE1 (MATa *leu2-3,112 ura3 his3 trp1*) was transformed to uracil prototrophy with the heat shock expression vector GA1695-

HSE1 (a kind gift of Dr P. Sorger, University of California, USA). GA1695-HSE1 contains a single heat shock element (HSE) sequence inserted in a disabled *CYC1* promoter fused to the *lacZ* gene (Sorger & Pelham, 1987). β -Galactosidase is not expressed at detectable levels in DBY747-HSE1 under normal conditions.

Overnight growth. DBY747-HSE1 was grown to exponential phase (2×10^6 cells ml⁻¹) in 8 × 60 ml aliquots in 250 ml conical flasks in a selection of yeast nitrogen base (YNB) media supplemented with histidine (20 mg l⁻¹), leucine (30 mg l⁻¹) and tryptophan (20 mg l⁻¹). Cells were grown at 25 °C or acclimatized in a 37 °C shaking water bath for a period of 16 h (during which time cells were diluted with YNB selective medium to maintain exponential growth). Samples were analysed for temperature profiles of HSE-*lacZ* induction and/or lipid profiles at the specified time points.

Commercial YNB medium. YNB (0.67%, w/v) without amino acids (Difco), in 'old' or 'new' plastic containers, containing 2.0% (w/v) glucose was used.

'Home-made' YNB. Stock solutions of the compounds detailed below were made up and autoclaved, or where specified, filter-sterilized. Stocks were stored at room temperature except where stated otherwise. Concentrations stated represent the final concentration in 1 l medium. Monobasic potassium phosphate (850 mg l⁻¹) and dibasic potassium phosphate (150 mg l⁻¹) were made up to a stock concentration of 100 × stated specification, and calcium chloride (100 mg l⁻¹) was made up to a stock concentration of 1000 × stated specification. Ammonium sulphate (5 g l⁻¹), sodium chloride (100 mg l⁻¹) and magnesium sulphate (500 mg l⁻¹) were each made up to a stock concentration of 50 × stated specification. L-Methionine (20 mg l⁻¹) was filter-sterilized. Dioctyl phthalate (serially diluted in ethanol) was added to the final concentration indicated, where appropriate.

The following vitamins were made up in a stock solution of 1000 × stated specification, filter-sterilized and stored in aliquots of 1 ml at -20 °C: biotin (20 µg l⁻¹), calcium pantothenate (2 mg l⁻¹), inositol (10 mg l⁻¹), pyridoxine hydrochloride (400 µg l⁻¹), thiamin hydrochloride (400 µg l⁻¹), folic acid (2 µg l⁻¹), *p*-aminobenzoic acid (200 µg l⁻¹), niacin (400 µg l⁻¹) and riboflavin (200 µg l⁻¹).

The following trace elements were made up in a stock solution of 1000 × stated specification, filter-sterilized and stored in aliquots of 1 ml at -20 °C: boric acid (500 µg l⁻¹), copper sulphate (40 µg l⁻¹), zinc sulphate (400 µg l⁻¹), potassium iodide (100 µg l⁻¹), manganese sulphate (400 µg l⁻¹) and sodium molybdate (200 µg l⁻¹). Ferric chloride (200 µg l⁻¹) was made up as a stock solution of 500 × stated specification, filter-sterilized and stored in aliquots of 1 ml at -20 °C.

Determination of temperature profiles of HSE-*lacZ*. Aliquots (10 ml) of exponentially growing cells were added to flasks containing 20 ml liquid YNB medium or 'home-made' YNB as specified, pre-heated to the stated temperature (in 250 ml conical flasks) in shaking water baths and subjected to a 10 min heat shock. The flasks were then placed in a 25 °C shaking water bath for a further 50 min to allow β -galactosidase expression from the induced transcripts.

Measurement of β -galactosidase activity from total cell extracts. β -Galactosidase activity was measured from a total cell extract as previously described (Chatterjee *et al.*, 1997). One representative set of experimental results (from at least three replicates) is presented for each temperature profile of

HSE-*lacZ* expression. The absolute level of β -galactosidase varied between experiments but these profiles were reproduced in repeated separate experiments.

Determination of the cellular lipid profile. Lipids were extracted and analysed using a modification of the method described by Hossack & Rose (1976) as previously described (Chatterjee *et al.*, 1997). The mean value and range from at least two separate experiments are presented in each figure.

HPLC. Comparative HPLC was carried out on an LKB system using a 2150 hplc pump and a LKB 2151 variable-wavelength monitor. The system was de-gassed with helium before a 10 μ l sample was loaded on a Li-Chromosorb column RP-18 (7 μ m particle size) and run on 35% acetonitrile/65% water (v/v) at a flow rate of 1 ml min⁻¹. Preparative HPLC was carried out under the same conditions except that repeated 10 μ l aliquots of a 10-fold concentrated YNB solution were added to the column until 0.5 ml of the 22 min peak had been collected.

RESULTS

An unexplained 3 °C shift in the temperature of maximal HSR in cells grown at 25 °C correlates with an increase in the level of unsaturated fatty acids in the cell

Heretofore, cells grown overnight at 25 °C and 37 °C were differentially sensitive to heat shock, with peak induction at 40 °C for cells grown at 25 °C and at 43 °C for cells grown at 37 °C (Fig. 1). Suddenly and inexplicably, the peak response of reporter gene expression was at 43 °C for cells grown at 25 °C, with an overall induction profile that had been previously restricted to cells grown at 37 °C (Fig. 1). Furthermore, the cellular heat shock sensitivity was strongly correlated with the lipid profiles of these cells. Whereas in the past (Chatterjee *et al.*, 1997), the cells grown at 25 °C contained 36% unsaturated fatty acids and those grown at 37 °C contained 65%, now the cells grown at 25 °C contained 73% unsaturated fatty acids (data not shown).

Fig. 1. Profile of heat shock induced β -galactosidase expression. ●, cells grown at 25 °C in 'old' YNB; ■, cells grown at 37 °C in 'old' YNB; ▲, cells grown at 25 °C in 'new' YNB.

Fig. 2. Percentage of cellular unsaturated fatty acids in cells grown in 'home-made' YNB containing various percentages of 'new' YNB.

Commercially produced YNB contains an extremely potent component

The aberrant lipid profile of the cells disappeared when YNB was prepared from its basic constituents to the specifications of the YNB commercially available from Difco. Cells grown at 25 °C in this 'home-made' YNB typically contained 44 ± 5% unsaturated fatty acids (column 0 in Fig. 2). This was significantly different from the 71 ± 7% found for cells grown in the current batch of commercially available YNB (column 100 in Fig. 2) but was, on the other hand, comparable to the 36 ± 4% found for cells grown at 25 °C in Difco's YNB in our previous experiments (Chatterjee *et al.*, 1997). An analysis of the lipid content of cells grown in 'home-made' YNB 'spiked' with various volumes of a 0.67% solution of the 'new' YNB revealed that this batch of YNB was affecting lipid metabolism. Surprisingly the 'lipid-modifying component' of the 'new' YNB exerted an observable effect on the level of unsaturated fatty acids in yeast cells when as little as 0.1 ml of the 0.67% solution was added to 59.9 ml of 'home-made' YNB (0.16% column in Fig. 2). Cells grown at this 1:600 dilution of the reconstituted YNB still contained 9% more unsaturated fatty acids than cells that had been grown in the absence of 'new' YNB.

HPLC identified a difference between 'old' and 'new' YNB

An HPLC analysis of reconstituted medium from an old container of YNB ('old' YNB) and from a new container of YNB ('new' YNB) revealed a significant difference in elution profiles. A peak found to elute isocratically with 35% acetonitrile/65% water (v/v) at a retention time of 22 min in the 'new' YNB did not appear in the 'old' YNB. Furthermore, a yeast bioassay revealed that cells exposed to as little as 100 μ l of this peak in 60 ml of 'home-made' YNB synthesized 31% more unsaturated fatty acids than untreated control cells (Fig. 3, compare columns E and C). No such increase was apparent when cells were grown in the presence of 100 μ l 22 min fraction from the 'old' medium (Fig. 3, column D). The percentages of unsaturated fatty acids found in cells

Fig. 3. Percentage of cellular unsaturated fatty acids in cells grown in 'old' YNB (A), 'new' YNB (B), 'home-made' YNB (C), 'home-made' YNB plus 'old' HPLC 22 min. sample (D), 'home-made' YNB plus 'new' HPLC 22 min sample (E).

Fig. 4. Profile of heat shock-induced β -galactosidase expression for cells grown in 'home-made' YNB in the absence of diethyl phthalate (■), in the presence of 36 μ M diethyl phthalate (▲), and in 37 °C acclimatized cells in 'old' YNB medium (●).

grown in 'old' and 'new' YNB medium are included for comparison (Fig. 3, columns A and B, respectively).

Mass spectroscopy identified dioctyl phthalate in the 22 min peak from the 'new' YNB; it was undetectable in the corresponding fraction from the 'old' YNB. An analysis of cells grown in 'home-made' YNB containing pure dioctyl phthalate (Sigma) confirmed that this compound was indeed capable of altering lipid metabolism and with it the cellular HSR.

Dioctyl phthalate increases the percentage of unsaturated fatty acids and decreases the cellular HSR sensitivity in yeast cells grown at 25 °C

Within limits, the percentage of unsaturated fatty acids present in the cells was a reflection of the concentration of dioctyl phthalate present in the medium (data not shown). The percentage of unsaturated fatty acids found in cells that had been exposed to concentrations of dioctyl phthalate up to and including 18 μ M was indistinguishable from the 41% found in cells grown in the complete absence of this compound. However, there was a 21% increase in the percentage of unsaturated

fatty acids in cells that had been exposed to 36 μ M dioctyl phthalate and an additional 15% increase, to 77%, in cells exposed to 72 μ M dioctyl phthalate. A further doubling of dioctyl phthalate to 144 μ M failed to increase the percentage of unsaturated fatty acids above 77%. The growth rates of these cultures were indistinguishable from one another but there was a significant decrease in the heat shock sensitivity of cells grown in the presence of dioctyl phthalate. The HSR profile of cells grown in the presence of 36 μ M dioctyl phthalate, the minimum level found to induce an increase in unsaturated fatty acids, is presented in Fig. 4.

Cells grown at 25 °C in the absence of dioctyl phthalate had minimal induction of β -galactosidase from the heat-inducible reporter gene at 38 °C, with peak induction at 40 °C. In contrast, this same temperature elicited only low-level β -galactosidase induction in cells containing elevated levels of unsaturated fatty acids (62%) after growth in the presence of 36 μ M phthalate. Furthermore, these cells underwent maximal induction at 43 °C; a temperature that elicited only a minimal response from cells grown in the absence of phthalate (Fig. 4). Cells grown at 37 °C in the 'old' medium exhibited maximal β -galactosidase induction at 43 °C (Fig. 4) and had 63% unsaturated fatty acids (Chatterjee *et al.*, 1997). Thus the HSR and lipid profiles of cells grown at 25 °C in 'home-made' medium containing 36 μ M dioctyl phthalate were the same as that found in cells that had been exposed to a 37 °C heat shock in 'old' YNB; a pattern that was also found in cells grown at 25 °C in 'new' medium (Fig. 1).

DISCUSSION

We have consistently found that the sensitivity of the yeast HSR system decreases as the level of unsaturated fatty acids increases in the cell. A typical example is provided in Fig. 1, where the 3 °C shift in the maximal HSR in cells grown at 25 °C and 37 °C was found to correlate with a 30% increase in the level of unsaturated fatty acids in the cell. Heat increases membrane fluidity; it is therefore counterintuitive that unsaturated fatty acids should be involved in desensitising a stress-response system that exists to prevent heat stress from compromising cellular function. It could be argued that this increase in lipid unsaturation helps to maintain the overall fluidity of the membrane in the face of the accumulation of the stress-associated membrane proteins Tip1, Tip1 homologues (Kondo & Inouye, 1991) and Hsp30 (Panaretou & Piper, 1992). However, the lipid and HSR profiles of the cells that had been grown at 25 °C in 'new' YNB in Fig. 1 cast doubt on this idea. These cells behaved in most respects like any other cells grown at 25 °C. They grew at the same rate and the heat shock reporter gene was off but their HSR and lipid profiles were the same as cells grown at 37 °C in previous experiments using 'old' YNB.

The first indication that the aberrant lipid and HSR profiles were associated with commercially available YNB medium was when cells grown in 'home-made'

YNB produced the same lipid and HSR profiles as cells grown in our earlier experiments. This can be seen in Fig. 2 where cells growing in 'new' YNB had 71% unsaturated fatty acids whereas cells growing in 'home-made' YNB only contained 44%. Some idea of the potency of the 'lipid modifying compound' can be gleaned from the fact that cells grown in a dilution series (up to 1:600) of 'new' YNB in the 'home-made' variety still produced more unsaturated fatty acids than that found in the control cells (Fig. 2). This suggested that the 'new' medium contained rather than lacked a lipid-metabolism-modifying constituent and indeed, a comparative HPLC analysis revealed a novel elution peak at 22 min that was unique to the 'new' YNB.

The results presented in Fig. 3 indicate that this fraction does indeed contain the mystery contaminant. Cells produced 73% unsaturated fatty acids when grown in 60 ml 'home-made' YNB in the presence of 100 µl of the 22 min fraction from the 'new' YNB, but only 42% unsaturated fatty acids when 100 µl from the equivalent peak of the 'old' YNB eluate was added (Fig. 3). Identified as dioctyl phthalate by mass spectroscopy, the results in Fig. 4 confirm the correlation between an increase in the level of unsaturated fatty acids in cells that have been grown in the presence of dioctyl phthalate and a decrease in their heat shock sensitivity. Cells grown at 25 °C in the absence of dioctyl phthalate contained 41% unsaturated fatty acids and responded maximally to heat shock at 40 °C, whereas cells grown in the presence of 36 µM dioctyl phthalate had 62% unsaturated fatty acids and maximal induction of the reporter gene at 43 °C (Fig. 4).

The mechanism by which phthalate has this effect on yeast cell lipid metabolism is unknown. In mammalian cells however, phthalate is known to induce peroxisome proliferation (reviewed by Latruffe & Vamecq, 1997). Peroxisomes serve a major function in fatty acid oxidation (reviewed by Small *et al.*, 1997) and indeed Rottensteiner *et al.* (1996) have shown that they are induced in *Saccharomyces cerevisiae* when oleic acid is supplied as the sole carbon source. It is unlikely however, that peroxisomes are induced under the growth conditions used here because peroxisomal structures and proteins are hardly detectable when glucose is present in the growth medium (Veenhuis *et al.*, 1987; Filipits *et al.*, 1993).

Phthalate is also known to increase the activity of protein kinase C (Bojes & Thurman, 1996) in mammalian cells. It is possible therefore, that phthalate could affect the equivalent protein in yeast affecting MAP kinase pathways such as the cascade found to be involved in cell-wall metabolism in *Saccharomyces cerevisiae* (Hunter & Plowman, 1997) or indeed by exerting an effect on yeast cell-cycle control (reviewed by Livneh & Fishman, 1997). It is not difficult to imagine subsequent knock-on effects occurring on lipid metabolism under such circumstances.

The details of how this molecule brings about these changes remains to be elucidated but given that a 1 h

exposure to 36 µM dioctyl phthalate failed to induce β -galactosidase expression from this heat shock reporter gene (data not shown), dioctyl phthalate treatment appears to confer a decrease in yeast HSR sensitivity without inducing the response. Moreover, the growth rate of the cells used in these experiments was the same regardless of whether they were grown in 0, 18, 36, 72 or 144 µM dioctyl phthalate, indicating that dioctyl phthalate does not significantly compromise overall cellular metabolism.

The results presented in this paper agree with earlier work from this laboratory that demonstrated that the percentage and type of fatty acid present in the cells determine the heat shock sensitivity of yeast cells (Chatterjee *et al.*, 1997). It also agrees with the work of Carratu *et al.* (1996), who decreased the HSR sensitivity of yeast cells grown at 25 °C by overexpressing the Ole1 gene product. Heat stress (Chatterjee *et al.*, 1997), overexpression of the Ole1 gene product (Carratu *et al.*, 1996), fatty acid supplements (Chatterjee *et al.*, 1997) and dioctyl phthalate (Fig. 4) all increase the percentage of cellular unsaturated fatty acids and decrease the sensitivity of the cellular HSR system, yet only the first of these is associated with induction of the HSR pathway. This indicates that even in the absence of stress-induced proteins, an increase in the percentage of unsaturated fatty acids is sufficient to decrease the HSR sensitivity of yeast cells.

Phthalate contamination of YNB prevented us from reproducing our earlier results for many months. It is indeed a strange irony that rather than undermining the original observations, it eventually provided even further support for the counterintuitive hypothesis that the down-regulation of the yeast 'heat-sensing mechanism' is intimately associated with an increase in the percentage of unsaturated fatty acids present in the cell.

ACKNOWLEDGEMENTS

We thank Alan Scott for expert technical assistance with HPLC and lipid analysis and Dr Virginia Bugeja for critically reading the manuscript.

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Received 11 April 2000; revised 25 May 2000; accepted 9 June 2000.