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Heat Shock Response and Cytotoxicity in C6 Rat Glioma Cells: Structure-Activity Relationship of Different Alcohols

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SUMMARY

In C6 rat glioma cells, the *n*-alcohols methanol, ethanol, propanol, and butanol and the aromatic alcohol phenol all induce heat shock proteins (HSPs) of high molecular mass (68, 70, 90, and 110 kDa) when applied for 1 hr. The lowest alcohol concentrations that induce HSP synthesis cause about 20% cell death, as determined by neutral red assay. HSP induction thus occurs at alcohol concentrations close to the highest tolerable dose. The cytotoxicity and the potential of alcohols to induce the synthesis of HSPs increase with chain length and are correlated with the lipophilicity of the alcohols. A clear structure-activity relationship is observed for both parameters. A calculation of the putative membrane concentrations of these alcohols reveals that cytotoxic effects (50% cell death) occur at nearly the same membrane concentration (approximately 0.2 M). This also holds true for the lowest HSP 68-inducing alcohol concentrations, but at a lower concentration (approximately 0.12 M). The activities of major proteinases are affected by both heat shock and alcohols. The effects of alcohols also depend on the lipophilicity of the alcohols. Effective concentrations again are close to the highest tolerable dose. The stress reactions measured in terms of significant changes in HSP synthesis and proteinase activity provide information about the mechanisms by which toxic agents act on the cell.

When cells are exposed to supraoptimal temperatures, a HSR is observed, which is particularly characterized by an increased or induced synthesis of several specific proteins (HSPs). The major HSPs show homologies among different organisms and a high functional significance as "molecular chaperons" under normal conditions as well as under stress conditions, and they are able to guide newly synthesized proteins into their correct conformations and cellular locations (for reviews, see Refs. 1–3). Many other cellular stressors, such as heavy metals, amino acid analogues, uncouplers, and substances affecting energy metabolism, also trigger the HSR, probably by increasing the amount of malfolded proteins within the cytoplasm (1). Malfolded proteins, however, not only play a role in the induction of heat shock genes but also are targets of the HSPs and may lead to changes of proteinase activities and protein turnover. Thus, changes in the rate of HSP synthesis or activities of proteinases may be useful markers to assess the amount of stress exerted on mammalian cells (4, 5). In this manuscript, the concentration-dependent effects of alcohols on HSP synthesis and proteinase activity were determined and compared with the cytotoxic potential of the alcohols. Cytotoxicity was investigated by means of cell viability tests, such as the NR uptake assay (6) and exclusion of trypan blue. Although alcohols have been reported to induce HSP synthesis in mammalian cells (7, 8), thus far a SAR has not been investigated.

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Materials and Methods

Chemicals. Chemicals were obtained from Sigma Chemicals Germany unless otherwise noted.

Cell culture. C6 rat glioma cells were grown in DMEM (Flow), supplemented with 10% fetal calf serum (GIBCO), at 37° in a humidified 10% CO_2 atmosphere.

Heat shock and alcohol shock. Cells (10^5/dish) were grown in 35-mm culture dishes for 3 days. A heat shock of 44° or an alcohol shock was then applied for 30 min or 1 hr, respectively. Cells were allowed to recover from the treatment for 2 hr, because in mammalian cells HSP synthesis is shown to begin approximately 1–2 hr after stress (3). In probing experiments we confirmed this also for C6 cells. The dishes in our experiments were not sealed. However, the loss of alcohol was only minor during the short exposure time of 1 hr, as was determined by measuring the weight loss of the dishes during the incubation.

***In vivo* labeling of proteins.** After shock, cells were washed with PBS and incubated in methionine- and cysteine-free DMEM (ICN Flow). Two hours after shock, cells were labeled for 4 hr with [^{35}S]

ABBREVIATIONS: HSR, heat shock response; HSP, heat shock protein; SAR, structure-activity relationship; NR, neutral red; DMEM, Dulbecco's modified Eagle medium; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CHO, Chinese hamster ovary; HSF, heat shock factor.

methionine (10 μCi/ml; ICN Flow). Cells were then washed with ice-cold PBS, harvested in sample buffer containing 1% SDS, and boiled for 5 min. The amount of trichloroacetic acid-precipitable radioactivity was determined in a Beckmann scintillation counter.

Electrophoretic analysis. For SDS-PAGE, aliquots of the cell extracts (200,000 cpm/lane) were loaded on 7.5–15% polyacrylamide gels and separated by SDS-PAGE as described (9).

Western blot analysis. For Western blotting, the proteins were separated by one-dimensional SDS-PAGE and were transferred to nitrocellulose membranes (BA83; Schleicher & Schuell) using a Trans Blot cell (Bio-Rad) (10). The blots were blocked with 0.2% Tween-20 in PBS for 1 hr at 25°. After removal of the blocking solution, primary antibody (monoclonal mouse anti-HSP 70, Sigma H-5147, 1/1000 dilution) in PBS/Tween-20 was added and left for 1 hr at 25°. Subsequently, the blots were washed with PBS/Tween-20. Secondary antibody [goat anti-mouse IgG (H+L) alkaline phosphatase conjugate, Bio-Rad 172–1015, 1/5000 dilution] in PBS/Tween-20 was added and, after 1 hr at 25°, the blots were washed with PBS/Tween-20. The immunocomplex was detected with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Boehringer Mannheim).

Native proteinase gels. Cells (5×10^5/dish) were grown in 35-mm culture dishes for 4 days at 37°, with one change of medium at day 3. After 4 days the cells were shocked for 1 hr with alcohol or elevated temperature (44°). Medium was then changed. After 8 hr of recovery, the cells were washed three times with 0.1 M Tris, pH 7.2, and harvested in the same buffer. Cells were disrupted by sonication (5 W, 15 sec). Proteinase activities were investigated in renatured gelatin gels (500 μg of protein/lane) as described (11). Optimal conditions for the activity of some proteinases were determined to be 12-hr incubation at 37° in 0.1 M glycine, 10 mM $CaCl_2$, pH 4.

Autoradiographs. Gels were vacuum-dried on a Whatman 3MM filter paper, placed on Kodak XR5 X-ray films, and kept at −80° during exposure. Autoradiographs were analyzed and quantified by means of video documentation equipment and evaluation program (Intas, Göttingen, Germany). HSP induction by alcohols was defined as the lowest concentration necessary for the induction of HSP 68 synthesis.

Protein content. Cells (5×10^4 cells, 0.5 ml/well) in a 24-well dish were grown for 3 days. The different alcohols were added to the cells in fresh medium for 1 hr. Cells were washed twice with PBS and grown again for 3 days. Protein content per dish was measured as described (12).

Trypan blue exclusion assay. Cells (5×10^4 cells/well) in a 24-well dish were grown for 3 days. The different alcohols were added to the cells for 1 hr in fresh medium containing 0.025% Trypan blue. Five hundred cells in each well were counted and the cytotoxicity was expressed as a percentage of surviving cells excluding the dye, in relation to an untreated control, which was set as 100%.

NR assay. Viable cells were assayed by NR uptake (6). NR (0.4 g) was diluted in 100 ml of water to give a stock solution. Before use, the stock solution was diluted 1/80 with DMEM containing 10% fetal calf serum, kept for 24 hr at 37°, and centrifuged at 2000 × *g*. Cells (2×10^4 cells/well) were cultured in 24-well plates for 3 days. Alcohol stress was applied for 1 hr. Each well was washed with 1.5 ml of PBS, and 1 ml of NR was added. After a 3-hr incubation at 37°, the cells were washed again with PBS and the dishes were dried "bottom up" for 10 min. Finally, 1 ml of a mixture of 50% alcohol and 1% acetic acid was added. After 10 min of agitation the absorbance at 540 nm was measured. Data are expressed as percentage of the untreated control. The highest tolerable dose is defined as the point at which the test agent exerts a 10% reduction in uptake (NR_{90}). IC_{50} (NR_{50}) is defined as the concentration leading to a 50% reduction in cell viability. Other indices to NR represent the corresponding percentage of cell viability.

Results

HSP synthesis. C6 rat glioma cells were exposed to different alcohols for 1 hr, were allowed to recover for 2 hr, and were then incubated with [^{35}S]methionine for 4 hr. Proteins were separated by one-dimensional SDS-PAGE, followed by autoradiography. This analysis revealed changes in the rates of synthesis (and/or degradation) of different protein species. In particular, we evaluated quantitatively the radioactive labeling of HSPs of higher molecular mass (68/70 kDa, 90 kDa, and 110 kDa) in relation to that of actin (42 kDa), as a standard that was set to 100%. We chose actin because it is known (3) and is evident from our own measurements that actin recovers rapidly after stress exposure and reaches control synthesis levels before labeling with [^{35}S]methionine.

All alcohols induce the high molecular mass HSPs. A clear induction of all HSPs is observed at concentrations leading to the induction of HSP 68, which is not synthesized constitutively. Slightly lower concentrations cause only slight increases in the synthesis rate of the constitutive HSPs, which are more difficult to quantify. HSP 68 induction thus appears to be a clearly visible induction parameter and was used for further studies.

A typical example of the response of HSP synthesis to different alcohol concentrations is given in Fig. 1a for butanol, as well as, for comparison, a typical pattern of HSP synthesis after heat shock (44° for 30 min). Within the group of the four aliphatic alcohols, butanol shows the strongest induction of HSP synthesis; an initial increase in the synthesis rates of HSPs is observed at 0.12 M (NR_{90}) and 0.13 M (NR_{75}), with maximal induction at 0.14–0.16 M (NR_{70} to NR_{55}). At these concentrations of butanol, the HSPs are synthesized at a rate close to that observed after heat shock (44° for 30 min) (Fig. 1). At even higher concentrations of butanol a rapid loss in cell viability hampered additional determinations of ^{35}S incorporation into HSPs. The quantified data of the autoradiographs (Fig. 1b) are based on the grey-level values of the protein bands, as visualized by a video camera and digitized by a computer system (see Material and Methods). The grey-level values of the HSPs were calculated with respect to the grey-level values of actin as a standard. Actin labeling remains constant over the concentration range tested.

A comparison of the different alcohols reveals the following order of lowest inductive concentrations for HSP 68: phenol (0.018 M) < butanol (0.14 M) < propanol (0.32 M) < ethanol (1.1 M) < methanol (2.2 M) (see Fig. 4a). Above the lowest inductive concentrations there is a small concentration "window" of inductive effects, as documented particularly for ethanol (1.1–1.3 M; see also below). Ethanol concentrations higher than 1.3 M inhibit the synthesis of HSPs.

Western blot analysis. When a monoclonal antibody against HSP 70 is applied, concentration-dependent expression of a particular member of the HSP 70 family is observed. Fig. 2 shows the levels of the constitutive HSP 70 and the inducible HSP 68 after treatment of the cells with different concentrations of ethanol. There is an obvious threshold of HSP 68 induction between 1.0 and 1.1 M for ethanol, with a maximum at 1.1 M. The degree of induction decreases until 1.3 M, whereas no induction can be measured at 1.4 M. The constitutive HSP 70 remains at the same level over the whole range of alcohol concentrations tested, although the level is slightly elevated, compared with the control.

With butanol, the increase of HSP 68 levels occurs at concentrations around 0.13 M and levels remain elevated up to at least 0.17 M, with a maximum at 0.15 M. Again, the HSP 70

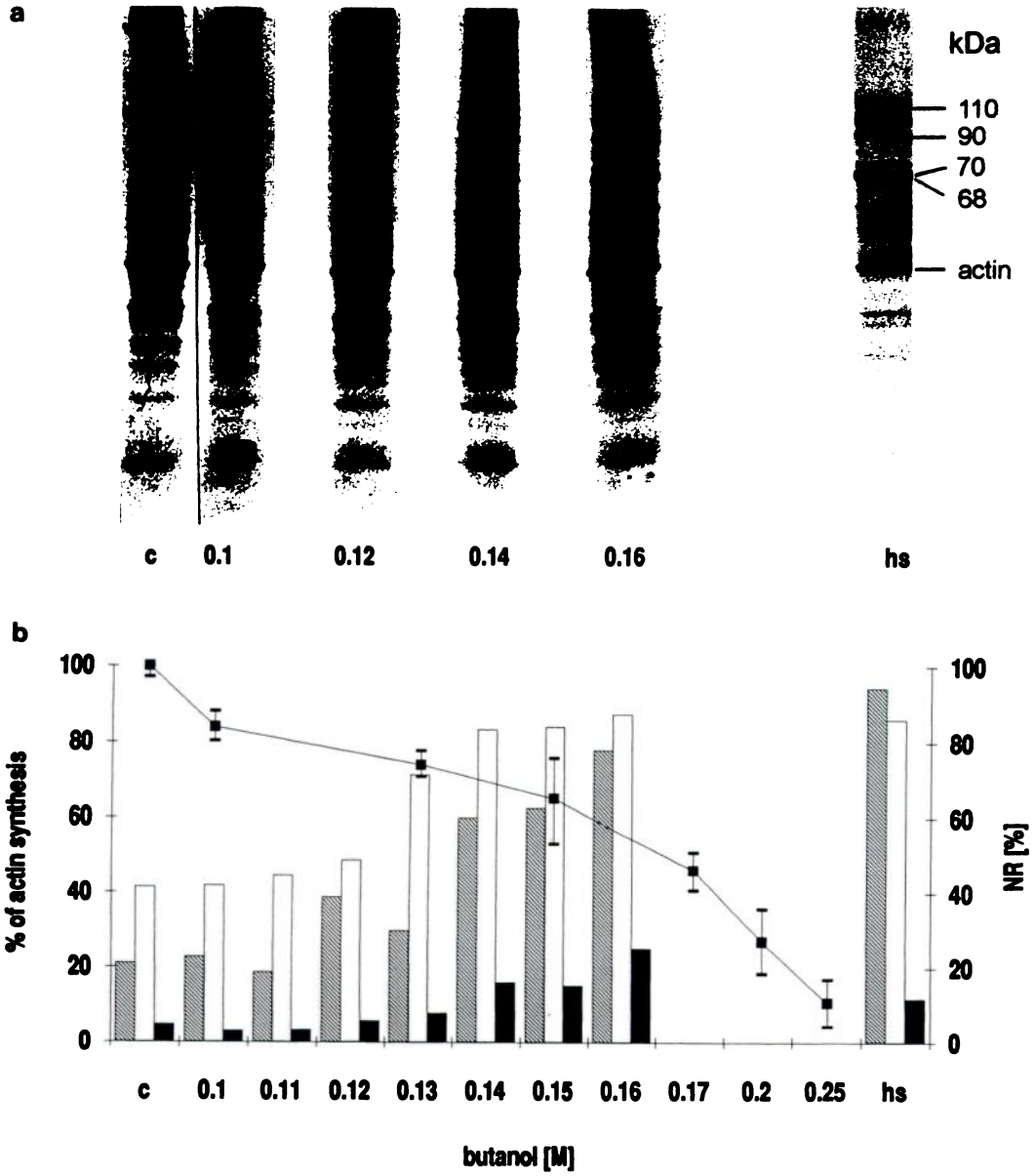

Fig. 1. Effect of butanol on HSPs and cytotoxicity in C6 cells, from analysis of HSP synthesis and cytotoxicity after treatment of cells with different concentrations of butanol for 1 hr. a, SDS-PAGE analysis of [^{35}S]methionine-labeled cell proteins. The cells were incubated in the presence of butanol (0.1–0.16 M) (*c*, control). After a 2-hr recovery in methionine/cysteine-deficient medium, 10 μCi of [^{35}S]methionine were added for 4 hr and cells were then harvested (*hs*, cells that were heat shocked for 30 min at 44° and labeled for 4 hr after a 2-hr recovery). The autoradiographs of cell lysates were run on a one-dimensional 7.5–15% polyacrylamide gradient gel. b, Quantitative evaluation of the relative labeling of the three major HSP bands (■, 70/68 kDa; □, 90 kDa; ■, 110 kDa), as percentage of actin labeling (*left ordinate*) at different concentrations of butanol (*abscissa*). *Curve with black squares*, NR uptake, as percentage of control (*right ordinate*). *Vertical bars*, standard deviations.

levels appear to be slightly higher in the range of concentrations tested (not shown).

Proteinase activity. C6 cell extracts were separated on gelatin-containing polyacrylamide gels. Proteinase activity was assayed in renatured gels, which were incubated for 12 hr at pH 4 (see Materials and Methods). Activity was attributed to white bands after staining of the gel. One region around 70 kDa changes after stress (Fig. 3); heat shock and 0.12–0.14 M butanol activate this proteinase, whereas little activity is observed in the control, with 0.1 M butanol, and with 0.16 M butanol. After heat shock, in particular, a decrease in the activity of a 30–40-kDa proteinase is observed, which is not as clearly visible after butanol treatment. Other proteinase bands, such as those of approximately 55 and 45 kDa, may also change after stress.

NR and trypan blue assays and protein content. To test the general cytotoxicity of the four aliphatic alcohols and phenol, the NR uptake and trypan blue exclusion were assayed after treatment with alcohol. In addition, we measured the protein content of the culture dishes 3 days after treatment. When the concentrations of the alcohols leading to 50% cell damage (NR_{50} or IC_{50}) or decrease in protein content per well are plotted versus the lipophilicity of the alcohols (expressed in terms of the membrane/buffer coefficient) (13), the cytotoxicity of the compounds significantly increases with their lipophilicity (methanol < ethanol < propanol < butanol < phenol) (Fig. 4a). Among the three test parameters, NR incorporation is the most sensitive, followed by protein content and trypan blue.

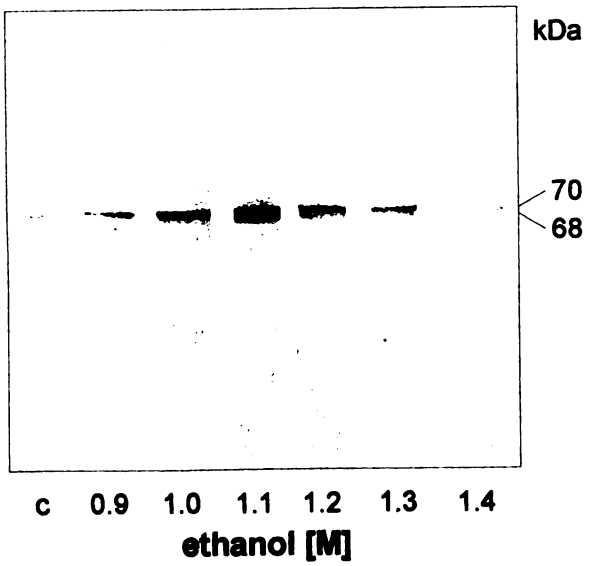

Fig. 2. Western blot analysis of HSP 70 and HSP 68, 15 hr after treatment of cells with different concentrations of ethanol (*c*, control). Proteins were separated by 10% SDS-PAGE and blotted, and the HSPs were determined by a monoclonal antibody against HSP 70/68.

When the cytotoxic response of C6 cells to alcohols, for example butanol, is compared with the HSR, the maximal induction of HSPs occurs below the NR_{50} value (Fig. 4b). This also holds true for the other alcohols (Fig. 4a), thus demonstrating that HSP induction generally occurs before the NR_{50} value. These results show that the induction of HSPs by alcohol is a response to considerable cellular damage.

The lipophilicity SAR of the different alcohols, as indicated in Fig. 4a, suggests that the alcohol partitioning in an nonpolar medium may be rather constant for a certain effect or damage. When the membrane concentrations of the alcohols with different carbon numbers are calculated from their partition coefficients (8), the values for NR_{50} and maximal HSP induction become rather similar (Fig. 4b); they vary between 0.13 and 0.25 M for NR_{50} and between 0.1 and 0.17 M for HSP induction. In this plot, methanol shows the highest cytotoxicity.

Discussion

Induction of HSP synthesis and thermotolerance. Ethanol has been shown to induce HSP synthesis in CHO cells (8, 14), Morris hepatoma cells (15), *Drosophila* (16), *Neurospora*,[1] and yeast (17). Ethanol concentrations necessary to induce a strong increase in the synthesis rates range between 1.0 M (CHO cells), 1.6 M (*Neurospora*), and 1.7 M (yeast) and correspond well to the concentrations applied in this study (1.1 M). These concentrations, however, relate to inducible HSPs, whereas smaller amounts of alcohol may increase the level of constitutive HSPs, as shown in Fig. 2. This increase may be due to a reduced degradation rate of constitutive HSPs during stress, rather than increased synthesis rates, as indicated by the effect of alcohols and heat on proteinase activities.

Ethanol was also shown to increase thermotolerance of CHO cells (18). Subsequent studies with different alcohols (propanol, pentanol, hexanol, and octanol) demonstrated that they exerted this effect at different concentrations (8). Those authors multiplied the thermotolerance-inducing concentration of a particular alcohol by its membrane/buffer partition coefficient and calculated similar membrane concentrations for the different alcohols, ranging between 0.12 and 0.32 M. From this calculation they concluded that membranes were the primary targets of alcohols and possibly involved in the induced thermotolerance.

Our data indicate that the capacity of alcohols to induce HSP synthesis is strongly correlated with their lipophilicity (Fig. 4a) and increases with the length of the carbon chain. A

[1] U. Meyer, P. Schweim, and L. Rensing, unpublished observations.

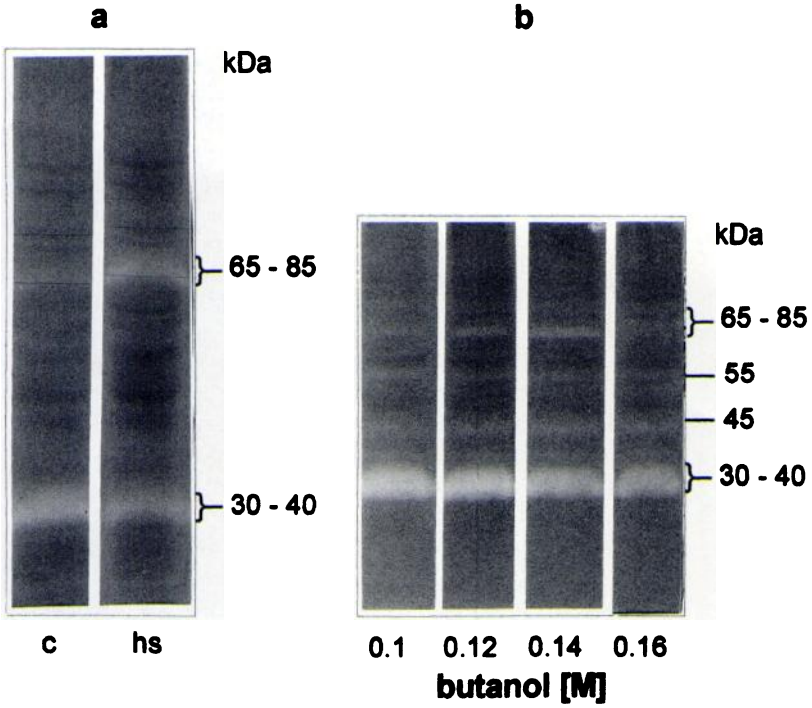

Fig. 3. Proteinase activities in renatured gelatin gels after heat shock (*hs*, 30 min at 44°; *c*, control) (a) and after treatment with different butanol concentrations (b). Cells were allowed to recover for 8 hr before proteins were separated by SDS-PAGE. After renaturation with Triton X-100, proteinase activities were determined after incubation of the gels at pH 4 and 37° for 12 hr.

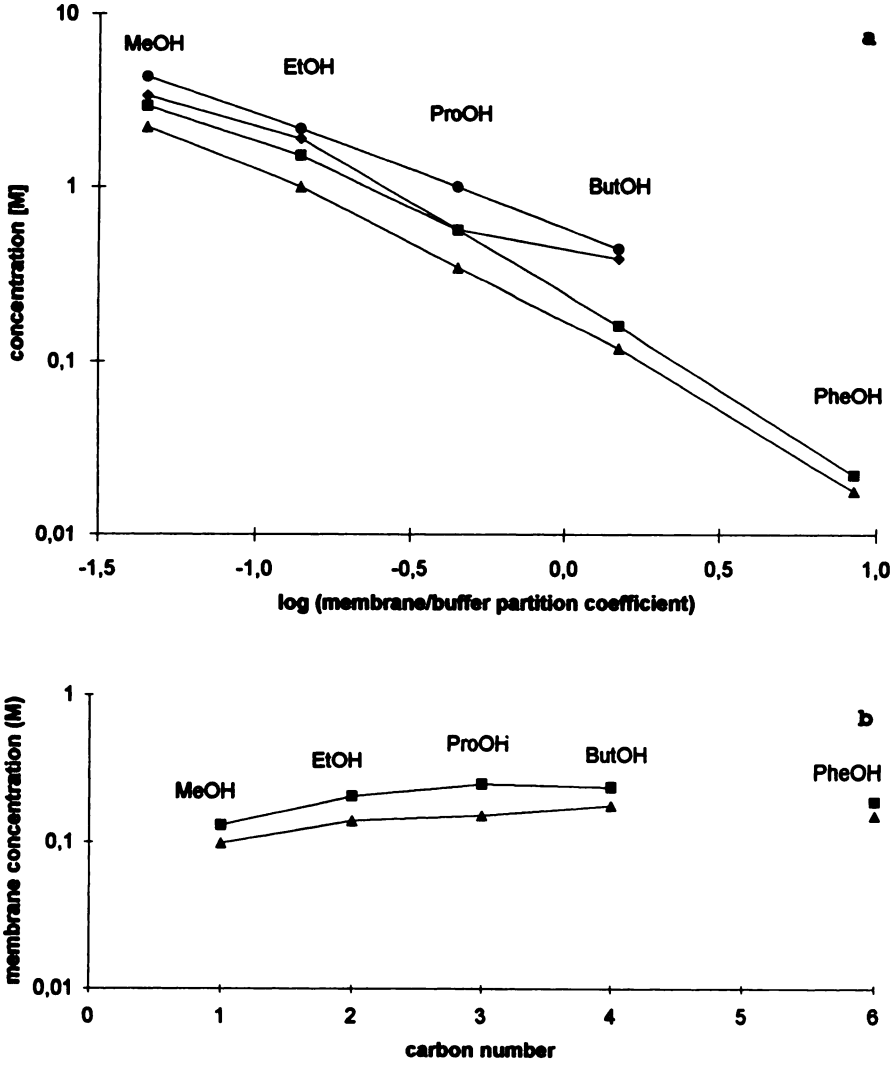

Fig. 4. Cytotoxicity and lowest inductive concentrations for HSP 68 synthesis of methanol (*MeOH*), ethanol (*EtOH*), propanol (*ProOH*), butanol (*ButOH*), and phenol (*PheOH*). a, ●, trypan blue exclusion; ♦, protein content 3 days after treatment; ■, NR assay; ▲, lowest inductive concentrations for HSP 68 synthesis after 1-hr incubation with different alcohol concentrations in the medium (*ordinate*). *Abscissa*, logarithm of the membrane/buffer partition coefficient for the different alcohols. b, ■, NR assay; ▲, lowest inductive concentrations for HSP 68 synthesis, depending on the calculated membrane concentrations of the different alcohols (*ordinate*). *Abscissa*, carbon number of the alcohols.

calculation of the hypothetical membrane concentrations of the different alcohols necessary to induce HSPs suggests a small range of concentrations (0.10–0.17 M). Hahn *et al.* (8) observed the induction of thermotolerance in the presence of propanol (0.7 M) for 30 min. In our experiments HSP 68 is induced after propanol treatment (0.34 M) for 1 hr. When the concentration of the alcohol is multiplied by the time, both approaches yield approximately the same values. These results again support the close correlation between induction of HSPs and thermotolerance. Which of the HSPs is primarily responsible for thermotolerance is not quite clear. Mutations of HSP 104 in yeast led to reduced survival at high temperatures and after ethanol treatment, whereas HSP 70 is important for the thermotolerance in *Drosophila* (19).

Induction mechanism. The proposed involvement of membranes in the HSP induction mechanism may be attributed to a changed flow of ions; particularly, cytoplasmic H^+ and Ca^{2+} concentrations have been shown to increase after heat shock (20–22). In addition, when C6 cells were incubated with 1.2 M ethanol, a considerable decrease of the intracellular pH was found by means of NMR spectroscopy.[2] Unequivocal evidence of H^+- or Ca^{2+}-induced changes in protein folding within the cell and subsequent activation of the HSFs responsible for the induction of heat shock genes is not yet available. However, *in vitro* experiments revealed activation of HSF by higher proton or calcium concentrations (23), indicating at least a promoting effect of these ion changes. In yeast, high and low extracellular pH values led to enhanced thermotolerance (24), again supporting the role of protons in the activation pathway.

An alternative explanation for the inducing effects of alcohols is their direct influence on protein folding. Alcohols may associate with lipophilic protein domains and thus stabilize abnormal conformations, which then may lead to enhanced HSP 70 binding. There are, for example, reports indicating alcohol-induced helical structures in peptides and proteins (25). Both explanations, i.e., ion-induced or direct effects on protein conformation, are compatible with current ideas about the activation of HSF by a dissociation of HSP 70 from the HSF under conditions of increased binding of HSP 70 to malfolded proteins (26).

Effects on proteinase activities. Applying the method of Heussen and Dowdle (11), we detected in C6 cells mainly proteinases active at low pH and in the presence of high calcium concentrations. The effects of alcohols and heat shock on the

[2] U. Neuhaus-Steinmetz, U. Pilatus, and L. Rensing, unpublished observations.

activity of proteinases reported here may be due to changes in their synthesis and degradation rates, as well as in their covalent modifications. Effects were observed at alcohol concentrations within the range leading to HSP induction; this, however, does not imply that any of these changes are related to the HSP system. On the other hand, alterations in the degradation system of the cell may lead to changes in the half-lives of particular proteins, including those of HSPs. Changes observed in the activity of two major proteinase bands (70 kDa and 35 kDa) may be due to different association/dissociation of subunits. These stress-induced alterations may be related to breaking of disulfide bonds caused by redox changes within the cell.

Alcohol toxicity and HSR. Alcohols are classical compounds with which to study SAR; the narcotic effects of alcohols, for example, clearly depend on their lipophilicity, as already noted by Meyer (27) and Overton (28) and recently summarized by Lipnick (29). In addition, other effects of alcohols, such as their general toxicity, show the same SAR (6, 30). A principally similar increase of toxicity with increasing lipophilicity can be observed in our experiments with NR and trypan blue assays, as well as with the overall protein synthesis rate. The induction of HSPs as well as the effects on proteinase activities showed the same dependency, but at slightly lower concentrations.

Hahn *et al.* (8) described a more complicated dependency, with a maximum effect of hexanol. However, such dependencies are difficult to evaluate, because of the rapidly decreasing water solubility of higher alcohols and because membrane concentrations were determined only hypothetically.

The use of HSR as a toxicity test has been proposed recently for *Escherichia coli* (4). Those authors saw an advantage in this test because the HSR appeared to be more sensitive, compared with growth inhibition. In C6 cells, the HSR is maximal at alcohol concentrations rather close to the IC_{50} values determined by the viability tests. This holds true for all three parameters tested; the increase of ^{35}S incorporation into HSP 68, the increase in its abundance, and the changes in the activity of a major proteinase. These reactions are thus elicited when cells are already damaged to an extent that ultimately may lead to cell death. The HSR is, however, a reaction not to all kinds of damage but perhaps only to perturbations affecting protein folding in the cell. Compared with other tests, such as NR or trypan blue exclusion, the HSR thus may give a more detailed picture of cell damage by a particular toxic agent.

Acknowledgments

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