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CHANGE IN MEMBRANE ORGANIZATION INDUCED BY HEAT SHOCK

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SUMMARY

In vivo change in membrane organization in adult rat liver cells induced by heat shock was studied by incorporating the fluorescent probes 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1-[4-(trimethy ammonio)phenyl]-6-phenyl-1,3,5-hexatriene (TMA-DPH) in these cells. DPH was essentially localized in the plasma membrane, as indicated by quenching of its fluorescence (over 90%) by 2,4,6-trinitrobenzenesulfonate (TNBS). Measurements of both fluorescence polarization and intensity indicate that there is an immediate change in membrane organization (order) following heat shock. The polarization changes of DPH and TMA-DPH incorporated into liver cell plasma membranes upon heat shock are in opposite directions. This result is attributed to the different locations of these probes in the membrane. This change in membrane organization could be a part of a general stress response which is then transmitted to other cellular organelles.

INTRODUCTION

Cells and tissues from a wide variety of organisms respond to environmental stresses such as heat by the rapid and transient expression of a highly conserved set of proteins called heat shock proteins (hsp) or stress proteins (1-6). Even though the stress response is rapid, leading to the expression of new proteins, the first organelle that senses stress is the cell membrane.

The capacity of adapting to hyperthermic stress is common to almost all living cells (7). Upon exposure to sub-lethal temperatures, cells acquire thermotolerance and survive for a longer period of time at elevated

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temperatures (8). Thermotolerance is the term generally used to refer to the transient, nonheritable state of resistance to the cytotoxic effects of subsequent hyperthermic treatments, which can be induced by a short exposure to sub-lethal temperatures. The involvement of heat shock proteins in thermotolerance is not very clear yet (7,8). A number of studies have focussed on how cells adapt to temperature induced alterations in the physical state of their membrane lipids. Homeoviscous adaptation of the membrane, *i.e.*, alteration of membrane lipid composition in order to maintain the same level of fluidity by replacing unsaturated fatty acids of the lipids by more saturated ones (9), has been shown to occur in bacteria (10,11), yeast (12), and eukaryotic cells (13,14). However, homeoviscous adaptation of membranes is a relatively slow process, often taking a few days (14,15).

In this paper, we report an immediate *in vivo* change in membrane organization of adult rat liver cells induced by heat shock by monitoring fluorescence changes of the incorporated probes, 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1-[4-(trimethylammonio)phenyl]-6-phenyl-1,3,5-hexatriene (TMA-DPH), in the membranes of these cells. DPH and its derivatives are the most extensively used probes to study order and dynamics in artificial and natural membranes (15-22). TMA-DPH is a derivative of DPH with a cationic moiety attached to the para position of one of the phenyl rings (23). These two probes were chosen since they monitor two different regions of the membrane bilayer (15,24,25). DPH is known to partition into the hydrophobic core of the lipid bilayer, whereas the amphipathic TMA-DPH gets oriented in the membrane bilayer with its positive charge located at the lipid/water interface, and its DPH moiety sensing the interfacial and headgroup regions of the membrane (15-17,23,26). Our studies employing these two probes indicate that there is an immediate change in membrane order of adult liver cells upon heat shock, as evidenced by changes in fluorescence intensity and polarization. Furthermore, this change in membrane order is not uniform across the depth of the membrane, but is position dependent.

MATERIALS AND METHODS

DPH and TMA-DPH were purchased from Molecular Probes (Eugene, Oregon, U.S.A.). Stock solutions of DPH in tetrahydrofuran and TMA-DPH in methanol were 1 mM. 2,4,6-trinitrobenzenesulfonic acid (TNBS) was from Pierce (Rockford, Illinois, U.S.A.). Neutralization of TNBS was carried out as described previously (27,28).

Adult Wistar rat liver cells were prepared by perfusing the liver with phosphate buffered saline (2 mM phosphate, 200 mM sodium chloride, 2.5 mM potassium chloride, pH 7.2). Single cell suspensions were made by gentle mechanical homogenization and cell viability was checked by trypan blue exclusion. Cell suspensions containing $2x10^6$ cells/ml in Dulbecco modified Eagle medium (DMEM) were incubated at normal (37°C) and heat shock (42°C) conditions. After 30 minutes of incubation at the two temperatures, DPH or TMA-DPH were added to give a final concentration of 1 mM (the amount of probe to be added was calculated based on the assumption that an average cell membrane contains $1x10^9$ lipid molecules) and incubated in 2 ml samples in triplicates at their respective temperatures for another 10 minutes to incorporate the probes in the membrane. At the end of the incubation, cells were washed free of the probe and resuspended in the same amount of buffer and absorbance and fluorescence were immediately measured. Fluorescence quenching experiments were performed as described previously (27,28). Duplicate samples of DPH labeled normal and heat shocked adult liver cells were incubated on ice in the presence of different concentrations (1.25-80 mM) of TNBS for 1 hour. TNBS was then washed off and fluorescence intensity measured. Unilamellar vesicles of dioleoyl-*sn*-glecero-3-phosphocholine (DOPC) were prepared by the ethanol injection method (29).

Fluorescence measurements were performed with a Hitachi F-4000 steady state spectrofluorometer using 1 cm path-length quartz cuvettes. Excitation and emission slits with a nominal bandpass of 5 nm were used for all experiments. All spectra were recorded using the correct spectrum mode. The excitation wavelength used was 358 nm in all cases. All fluorescence measurements were done at 23°C. To reverse any photoisomerization of DPH, samples were kept in dark in the fluorimeter for 30 seconds before the excitation shutter was opened and fluorescence measured (30). Background intensities of samples in which fluorophores were omitted were negligible. Polarization measurements were performed using Hitachi polarization accessory. Polarization values were calculated from the equation (31):

$$P = \frac{I_{VV} - GI_{VH}}{I_{VV} + GI_{VH}}$$

where I_{VV} and I_{VH} are the measured fluorescence intensities with the excitation polarizer vertically oriented and the emission polarizer vertically and horizontally oriented, respectively. G is the grating correction factor and is equal to I_{HV}/I_{HH}. The polarization values reported in Table II represents the average of at least twelve measurements in each case.

RESULTS

Time course incubation experiments with adult liver cells using DPH and TMA-DPH showed that 10 minutes incubation (at 37° or 42°C) with the probes added to the cells which were preincubated for 30 minutes at their

respective temperatures (37° or 42°C) was appropriate for fluorescence measurements. We have previously demonstrated that . hour incubation of adult liver cells at 42°C induces heat shock response leading to the synthesis of heat shock proteins (32). Since incubation for this length of time gave relatively steady fluorescence, these conditions were used in all experiments.

Partitioning of fluorescence probes into intracellular organelles could be a complicating factor when fluorescence methods are employed to evaluate the fluidity parameters of plasma membranes of intact cells. Grunberger *et al.* (27) developed a method for resolution of fluidity parameters under such conditions. This method is based on the selective quenching of fluorescence from the plasma membrane by the impermeable quencher TNBS. To confirm that DPH was localized exclusively in the plasma membrane under our experimental conditions, DPH labeled adult liver cells were incubated with various concentrations of the quencher TNBS and the *in vivo* fluorescence measured. Results presented in Table I show that more than 90% of DPH fluorescence was quenched by TNBS. This clearly demonstrates that DPH is essentially localized in the plasma membrane.

TABLE I

Fluorescence quenching of DPH incorporated into adult rat liver cells at 37° and 42°C using TNBS*

Concentration of TNBS (mM)	Percent quenching at 37°C	42°C
0	0	0
1.25	69	6[illegible]
2.5	74	7[illegible]
5	82	8[illegible]
10	85	8[illegible]
20	89	9[illegible]
40	92	9[illegible]
80	93	9[illegible]

* All experiments were done at 23°C. See Materials and Methods for other details.

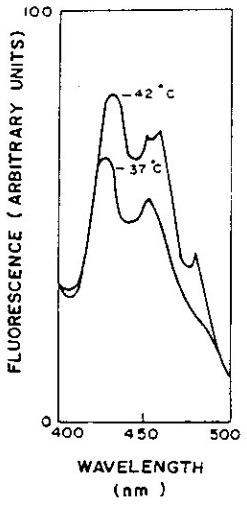

Figure 1. Corrected fluorescence emission spectra of DPH incorporated into adult rat liver cells at 37° and 42°C. See Materials and Methods for other details.

Figure 1 shows the corrected fluorescence spectra of DPH incorporated into rat liver cells at 37° and 42°C. These spectra are characteristic of membrane-bound DPH molecules with the emission maxima around 428 nm (23). As is seen from the figure, there is an increase in fluorescence in the heat stressed samples. This could be because of differential uptake of DPH at these two temperatures. However, this possibility is ruled out since cells in which DPH has been taken up at 37°C, when incubated at 42°C for 30 minutes, also showed a similar increase in fluorescence. In addition, the optical densities of the samples at 350 nm were identical at 37°C and 42°C indicating that the amount of probe incorporated was the same.

Figure 2 shows the corrected fluorescence spectra of TMA-DPH taken up by liver cells at 37° and 42°C. In this case also, there is an increase in fluorescence at 42°C. However, the magnitude of this increase is somewhat less than DPH. This differential increase in fluorescence in the two cases possibly reflect the different locations of these probes in the membrane (see later). We interpret these changes in fluorescence as a result of microenvironmental changes occuring in the immediate vicinity of these fluorophores, resulting from a change in membrane organization induced by heat shock.

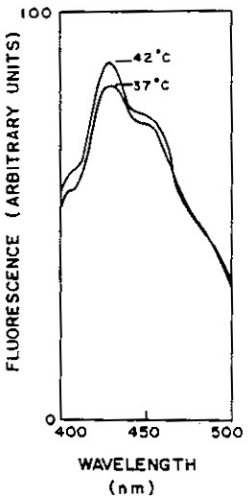

Figure 2. Corrected fluorescence emission spectra of TMA-DPH incorporated into adult rat liver cells at 37° and 42°C. All other conditions are as in Fig. 1.

To further characterize this change in membrane organization, we measured fluorescence polarization of DPH and TMA-DPH incorporated into liver cells at 37° and 42°C. The degree of depolarization of the emitted fluorescence from such membrane-bound probes depends on the rotational mobility of the probe in the timescale of fluorescence (typically nsecs) in the given environment. If the fluorophore undergoes rapid rotational motion giving rise to substantial change in orientation during the lifetime of the excited state, its polarization is reduced. The fluorescence polarizations of DPH and TMA-DPH incorporated into liver cells at 37° and 42°C are shown in Table II. As is seen from the table, the polarization of DPH increases upon heat shock. This is attributed to the change in membrane organization earlier evidenced by a change in fluorescence intensity (discussed above) brought about by heat shock. Thus, the rotational mobility of the DPH molecules decreases (as deduced from an increase in fluorescence polarization) upon heat shock. This increase in polarization would indicate an overall increase in the rigidity of the membrane hydrocarbon region. There is also a change in the polarization of TMA-DPH induced by heat shock indicating a change in membrane order.

It is interesting to note that, unlike DPH, the polarization of TMA-DPH decreases, implying that the region of the membrane monitored by TMA-DPH becomes less rigid following heat shock. Such differential ordering effects in various regions of the membrane, as monitored by DPH and TMA-DPH, have been previously reported (15,20,21). Although the exact significance of such localized ordering and disordering is not very clear, it is known that DPH and TMA-DPH have different orientations in the membrane and the DPH moiety in these two cases is located in different regions of the bilayer (15,23-25). The precise location of DPH in the membrane is still uncertain although it is known that it partitions into the hydrophobic core of the membrane (16,17). Fluorescence polarization of DPH in membranes thus provides an overall measure of the structural order in the interior of the membrane (17,24). TMA-DPH, on the other hand, monitors the interfacial and headgroup regions of the membrane and is oriented in the bilayer with its long axis perpendicular to the membrane plane. Accordingly, the fluorescence polarizations of TMA-DPH in membranes is always considerably higher than that of DPH (see Table II and 15,20,21). This is reflective of the higher degree of order in the glycerol backbone region compared to the fatty acyl chain region of the membrane.

TABLE II

Fluorescence polarizations of DPH and TMA-DPH incorporated into adult rat liver cells at 37° and 42°C*

Probe	Polarization values (P) for incubation at 37°C	42°C	ΔP
DPH	0.149 ± 0.009	0.165 ± 0.008	0.016
TMA-DPH	0.296 ± 0.017	0.260 ± 0.029	0.036

* All polarization measurements were done at 23°C. See Materials and Methods for other details.

In a control study (not shown), we measured the polarizations of DPH and TMA-DPH incorporated into unilamellar vesicles (liposomes) made with DOPC. Fluorescence was measured after incubating the liposomes at 37° and 42°C using the same protocol used for the liver cells. We did not observe any significant directional change of fluorescence polarization with heat shock in these cases.

DISCUSSION

Temperature changes are known to cause a number of physiological and biochemical changes in cells and tissues. Induction of heat shock proteins due to thermal as well as other types of stress has been well characterized in a variety of cell types and organisms (1-6). The plasma membrane is thought to be the primary site of heat shock effects. Their unique state as a highly ordered but permanantly changing fluid makes them suitable targets of heat shock effects. The effect of heat shock on plasma membranes, the organelle that first senses and responds to the applied stress, is not yet clearly understood. In an earlier study (14), it was reported that changing the growth temperature of plateau phase Chinese hamster ovary cells resulted in changes in membrane fluidity, detected by fluorescence polarization of DPH incorporated into these cells. This was attributed to adaptive changes in cholesterol to phospholipid molar ratio. However, this change in membrane organization was due to long (days) exposure of cells to sub-lethal temperatures. In such studies of homeoviscous adaptation (14,15), the membranes were isolated after the cell adapted to the new temperature and DPH was incorporated into the isolated membranes. The present investigation is aimed at studying *in vivo* changes in plasma membrane organization immediately following heat shock.

Our results indicate a change in membrane organization of adult liver cells immediately following heat shock. We have previously demonstrated that heat shock for this length of time induces expression of heat shock proteins in liver cells (32). Other workers have recently reported that heat shock induces aggregation of membrane proteins (33) and makes pores in membranes (34). The change in membrane organization reported here is likely to be coupled to these effects of heat shock. The immediate change in plasma membrane organization could be crucial for providing the optimal membrane

environment necessary for carrying out critical membrane functions, and could be a part of a general stress response which is then transmitted to other cellular organelles.

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