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Review

Plasma membranes as heat stress sensors: From lipid-controlled molecular switches to therapeutic applications☆☆

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ABSTRACT

The classic heat shock (stress) response (HSR) was originally attributed to protein denaturation. However, heat shock protein (Hsp) induction occurs in many circumstances where no protein denaturation is observed. Recently considerable evidence has been accumulated to the favor of the "Membrane Sensor Hypothesis" which predicts that the level of Hsps can be changed as a result of alterations to the plasma membrane. This is especially pertinent to mild heat shock, such as occurs in fever. In this condition the sensitivity of many transient receptor potential (TRP) channels is particularly notable. Small temperature stresses can modulate TRP gating significantly and this is influenced by lipids. In addition, stress hormones often modify plasma membrane structure and function and thus initiate a cascade of events, which may affect HSR. The major transactivator heat shock factor-1 integrates the signals originating from the plasma membrane and orchestrates the expression of individual heat shock genes. We describe how these observations can be tested at the molecular level, for example, with the use of membrane perturbers and through computational calculations. An important fact which now starts to be addressed is that membranes are not homogeneous nor do all cells react identically. Lipidomics and cell profiling are beginning to address the above two points. Finally, we observe that a deregulated HSR is found in a large number of important diseases where more detailed knowledge of the molecular mechanisms involved may offer timely

Abbreviations: AA, arachidonic acid; APAP, acetaminophen; aSMase, acid sphingomyelinase; ATP, adenosine triphosphate; BA, benzyl alcohol; BM, bimoclomol; CaMKII, calmodulin kinase II; Cdase, ceramidase; CHO, Chinese hamster ovary; CHOL, cholesterol; CRA, crotonaldehyde; DAG, diacylglycerol; DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; EGFR, epidermal growth factor receptor; ERK1/2, extracellular-signal-regulated kinase; FRET, fluorescence resonance energy transfer; GCS, glucosylceramide synthase; GFP, green fluorescent protein; GFR, growth factor receptor; GlcCer, glucosylceramide; GPI, glycophosphatidylinositol; GR, glucocorticoid receptor; GSK3, glycogen synthase kinase-3; HNE, 4-hydroxynonenal; HSF1, heat shock factor 1; HSP, heat shock protein; HSR, heat shock response; IP3, inositol trisphosphate; LB, luria broth; Ld, liquid disordered; Lo, liquid ordered; LPA, lysophosphatidic acid; LPA, alpha lipoic acid; LPC, lysophosphatidylcholine; LPS, lysophosphatidylserine; MALDI, matrix-assisted laser desorption/ionization; MAPK, mitogen-activated protein kinase; MBCD, methyl-β-cyclodextrin; MD, molecular dynamics; MPS, membrane physical state; mTOR, target of rapamycin; NADA, *N*-arachidonoyl-dopamine; NPN, 1-N-phenylnaphthylamine; PhA, phenethyl alcohol; PHB, poly-(R)-3-hydroxybutyrate; PI3K, phosphatidylinositol-3-kinase; PI4P, phosphatidylinositol 4-phosphate; PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinositol-3,4,5-triphosphate; PKA, protein kinase A; PKC, protein kinase C; PLA_2, phospholipase A_2; PLC, phospholipase C; PLD, phospholipase D; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; S1P, sphingosine-1-phosphate; SFA, saturated fatty acids; SGT, glucosyltransferase; SK1, sphingosine kinase 1; SM, sphingomyelin; SPC, sphingosylphosphorylcholine; TIRF, total internal reflection fluorescence; TOCCSL, Thinning Out Clusters while Conserving the Stoichiometry of Labeling; TRP, transient receptor potential channel; UFA, unsaturated fatty acids; YFP, yellow fluorescent protein

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Contents

1. Introduction: new insights into the mechanism of heat shock response activation

All organisms can be exposed to environmentally-imposed stressors such as elevated temperatures or UVB irradiation, physiological stressors such as rapid cellular proliferation, or pathophysiological stressors caused by infection or inflammation. Even normal developmental or nutritional changes exert stress as systems temporarily depart from and try to re-establish homeostasis. Responsiveness to diverse stressors may arise from the most striking and common impact of stress: it deforms and may damage the cell constituent molecules such as membrane lipids, proteins or nucleic acids [1]. If unmitigated, such stressful conditions lead – among others – to protein misfolding and aggregation, membrane remodeling and raft disruption, cellular dysfunction and cell death. Universally-conserved stress proteins can be regarded as the minimal stress proteome. Functional analysis of the minimal stress proteome is the best source to point for the major aspects of cellular stress response. Hsps were first identified as protectors of the integrity of proteins and as guardians of the cell against stress. The cytoprotective functions of these Hsps rest upon more than one molecular function; protection from stress requires molecular chaperone activity to block and repair protein unfolding as well as specific inhibition of effector proteins in the programmed cell death pathways, capabilities embedded within the sequences of Hsps [2]. More recently – with active participation of our laboratories – these proteins were shown to possess a third function, which is to migrate away from the "canonic" intracellular sites of action to novel locations including the plasma membrane, inner lysosomal membrane or even the extracellular compartments [3].

Due to their multiple and vital functions, Hsps play a fundamental role in the pathology of several human diseases. Aberrantly high levels of certain Hsp classes are characteristic in cancer cells and the converse situation applies typically for aging, type 2 diabetes or for many neurodegenerative diseases. Accordingly, the mechanism of how stress conditions (including pathophysiological states) can be sensed and transduced to regulate the expression of Hsp-genes is of key importance [4].

Since many of the stressors are believed to cause the accumulation of abnormal proteins, sensing high temperature has been attributed in the past mostly to the denaturation of thermolabile proteins. But our studies (and recently others, as well) strongly suggest that cells have the ability to sense and respond to stress signals also through the activation of membrane-associated signal transduction pathways. In our 1998 article for *Trends in Biochemical Sciences*, we first discussed how the membrane's physical state could influence gene expression and, hence, responses to stress [5]. Since then, knowledge of the interactions between the plasma membrane and the gene-expressing machinery has increased considerably, as has the realization that membranes display microheterogeneity. Our concept of the plasma membrane has changed considerably as our understanding of membrane microdomains and lipid rafts has increased [6]. Because the raft structure is strongly dependent on the thermally-controlled lipid-phase behavior, we assume that even mild changes in temperature could result in altered fluidity and consequently, redistribution and modified activity of potential stress-sensing/signaling proteins within these subdomains. The universal induction of cell stress response in most various organisms (from bacteria and algae to yeast, plant and mammalian cells) by

the simple treatments with nonproteotoxic membrane fluidizers strongly suggests that subtle changes in the surface membranes are the most upstream events of the temperature-sensing and signaling. Network theory is increasingly becoming a prevailing paradigm to describe the diverse cellular functions under both normal and disease or stressed states, as well. Hsp chaperones are known to form complex interaction networks (the "chaperome") with each other and their protein partners [7]. Obviously, this hierarchical cellular structure should be further extended by acknowledging, that a subpopulation of Hsps is temporarily or permanently membrane-associated and, that the individual membrane lipid molecules can be active participants of the folding of membrane proteins [3].

Proof-of-principle examples are available for successful drug development boosting components of the heat shock system and alleviating disease. However, the efficacy of these drugs is cell- and tissue-specific as it depends on the context of existing, active and inactive signaling pathways. Our ultimate focus is to develop membrane interacting substances (drugs acting on the base of "membrane lipid therapy") to specifically target disease-relevant heat shock genes [8].

2. Evidence on structural changes in the plasma membrane during fever-type heat shock

Recently, we made an interesting observation: when studying the behavior of GPI-anchored proteins in the plasma membrane in living Chinese hamster ovary (CHO) cells, we found that the proteins diffuse as dimers, at least to a significant extent (about 30%) [9]. The association was extremely stable (over seconds), required the presence of cholesterol in the membrane, and showed a strong temperature-dependence. In this paragraph, we provide our tentative interpretation of the observation, and discuss its links to the current literature.

Our approach is based on imaging single fluorescently labeled protein molecules in the live cell plasma membrane. In general, the image of a single dye molecule encodes its position (equivalent to the center of mass of the signal distribution) and its brightness (the total number of counts in the spot). In simple terms, n colocalized dye molecules are n-times brighter than one molecule, therefore the brightness can be used for determining the stoichiometry of a fluorescently labeled complex [10]. This approach works nicely for proteins at low surface density in the plasma membrane, so that individual molecules can be resolved as well-separated spots [11]. In most situations, however, higher surface densities preclude a direct analysis.

For such cases we have devised a method acronymed TOCCSL (Thinning Out Clusters while Conserving the Stoichiometry of Labeling) [12,13]. Briefly, a strong laser is used to photobleach all fluorophores within a well-defined area, so that complexes are either switched off completely (within the photobleached area) or left intact (outside of this area). Next, diffusional motion of the probe molecules leads to recovery of fluorescence. At the very onset of this recovery process, the first protein molecules or complexes moving into the photobleached area can be observed as well-separated fluorescent spots. Fig. 1 shows a typical TOCCSL experiment. The selected cell shows a uniform and homogeneous membrane staining (Fig. 1A,B). Immediately upon photobleaching via a rectangular field-stop, the fluorescence signal is completely erased within the selected area (Fig. 1C), and is slowly repopulated from the edges of the field-stop (Fig. 1D); in the center of the area, individual spots are analyzed for their brightness.

In the referred study, we were interested in the association behavior of GPI-anchored proteins within the plasma membrane. When treating cells with detergent, GPI-anchored proteins were described to partition preferentially in the raft fraction [14–17], so we speculated that these proteins may provide a route toward deciphering the enigmatic rafts [6,18]. For that, we fused monomeric GFP [19] to the GPI-anchor of the folate receptor [20] and expressed the construct in CHO cells. The TOCCSL data revealed that about 20–40% of the observed spots corresponded to mGFP-GPI dimers (Fig. 1E). Dimers were stable at least over the time-scale of the experiment of a few seconds. Depleting membrane cholesterol abolished the dimers completely. In addition, a mild fever-type heat shock substantially reduced the dimer fraction (Fig. 1F).

The data are surprising: the high stability of the complexes appears unexpected for a lipid-mediated cohesion [21–23]. There are ideas that the coupling to actin could stabilize per se transient clusters by pinning them to the cortical cytoskeleton [21,23,24]; the clusters observed in our experiments, however, were highly mobile (diffusion constant D ~ 1.3 $\mu m^2/s$) and therefore cannot be explained solely by interactions with the cytoskeleton. Indeed, our observations are in line with observations of other researchers. For example, the lab of Kusumi reported the formation of homodimers of GPI-anchored proteins, which could be directly followed by single molecule tracking [25]. From studying various chimeras, the interactions could be ascribed to contributions both from the lipid-environment and the protein ectodomains, resulting in a lifetime of the protein complexes of ~200 ms, somewhat shorter than in our study.

It is quite enlightening to follow up on the idea of multiple contributions to the binding process. Let us consider here the following simple binding reaction:

$$A + B \underset{k_-}{\overset{k_+}{\rightleftharpoons}} A \cdot B \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} AB.$$

A and B denote the two binding partners which first form an encounter complex $A \cdot B$, before the reaction proceeds to the bound complex AB. k_+, k_{on}, k_-, and k_{off} describe the forward and backward rate constants (here all in units of s^{-1}). The two-dimensional diffusion-limited rate for molecule A to collide with molecule B can be estimated by

$$k_+ = 2\pi c_B \left[\frac{D_A}{ln\left(\frac{1}{r\sqrt{\pi c_B}}\right)} + \frac{D_B}{ln\left(\frac{1}{r\sqrt{\pi c_A}}\right)} \right]$$

where D_A and D_B denote the diffusion coefficients, c_A and c_B the surface densities of substance A and B, and r the radius of the encounter complex [26,27]. The rate for dissolution of the encounter complex is given by $k_- = 2[D_A + D_B]/r^2$ [28]. For this two-stage reaction, the overall equilibrium is characterized by the constant $K_{total} = \frac{k_+}{k_-} \cdot \frac{k_{on}}{k_{off}} = K_{encounter} \cdot K_{binding}$; the two stages – formation of the encounter complex and binding – can therefore be factorized.

Next, two interacting proteins generally consist of multiple binding motifs, each contributing with ΔG_i to the total interaction energy $\Delta G_{binding} = \sum_i \Delta G_i$. This converts to a product when calculating equilibrium constants $K_{binding} = \prod_i K_i$, with $K_i = exp\left(-\frac{\Delta G_i}{RT}\right)$, R denoting the gas constant, T the temperature. In other words, the full binding process can be described by a product of equilibrium constants.

For simplicity, we consider here the three main contributions to the interaction of two GPI-anchored proteins: the encounter complex formation $K_{encounter}$, the protein ectodomain $K_{ectodomain}$, and the lipid environment K_{lipid}, with $K_{binding} = K_{ectodomain} \cdot K_{lipid}$. A few conclusions can be drawn immediately:

- *Membrane-anchorage allows for oligomerization of ectodomains with low interaction energy*. Due to the high k_+ in two dimensions, the formation of encounter complexes is favored ($K_{encounter}$ becomes large). In addition, the lipid environment can contribute by stabilizing the interaction (K_{lipid} may get large). Finally, the limited rotational mobility of membrane proteins can further increase k_{on} [29].
- *Multiple parameters may modulate the interaction*. There are indications that the natural plasma membrane shows phase separation into nanoscopic liquid ordered (Lo) and liquid disordered (Ld)

phase [30,31]. Preferential partitioning of the protein of interest to one of the two phases will therefore yield a higher surface density within the preferred phase, when compared to a uniform distribution of the protein. For our case of a GPI-anchored protein, preferential lo-phase partitioning will raise the local surface density of the GPI-protein; consequentially, k_+ and thus $K_{encounter}$ will be high. The lower diffusion constants within the Lo phase will hardly affect the binding equilibria, as they reduce on- and off-rate proportionally. In addition, the local lipid composition within the preferred Lo phase may alter the binding reaction directly via ΔG_{lipid}. For example, locally increased cholesterol and ganglioside GM1 concentration in the Lo phase [32] may assist in the stabilization of complexes of GPI-anchored proteins.

Let us summarize these thoughts in view of a cell's capability to sense temperature. First, it is highly plausible that lipid membranes generally act as sensors for changes in the environment, which affect K_{total} by changed equilibria of membrane protein interactions. Changed protein interactions would naturally influence a variety of signaling pathways that involve cell surface receptors. Second, temperature, in particular, has a strong influence on membrane properties such as the phase state of multi-component mixtures. Plasma membrane vesicles, for example, phase-separate into micrometer-sized Lo–Ld coexistence regions at room temperature, which shrink to nanoscopic domains of fluctuating size at physiological temperature [33]. Third, temperature-dependent nanoscopic rafts may thus stabilize interactions of GPI-anchored or other membrane proteins by increasing $K_{encounter}$ and K_{lipid}. A rise in temperature would result in the disassembly of rafts, and in a homogeneous distribution of the membrane proteins. Consequentially, $K_{encounter}$ and K_{lipid} will be lowered, resulting in a decreased binding rate of the membrane proteins — as observed in our study (Fig. 1F).

3. TRP channels and temperature transduction

Transient receptor potential (TRP) channels constitute a large family of cationic channels. First identified in *Drosophila* (reviewed by [34]), TRP channels play important roles in sensory physiology, including the transduction of chemical, temperature and mechanical stimuli [35,36]. Many TRP channels act as polymodal transducers, responding to different forms of energy, as a result of cooperative allosteric interactions in their gating [37].

Several TRP channels are expressed in terminals of primary sensory neurons covering the skin and mucosa lining the surface of the body (cornea, mouth, nasal epithelium). Here, they can be exposed to rapid temperature fluctuations. In regard to temperature sensing, some TRP channels show remarkable temperature sensitivity, with temperature coefficient (Q10) values larger than 20 [38,39], compared to values between 1 and 3 which are typical for other proteins. Their activation results in local depolarisations and the generation of action potentials

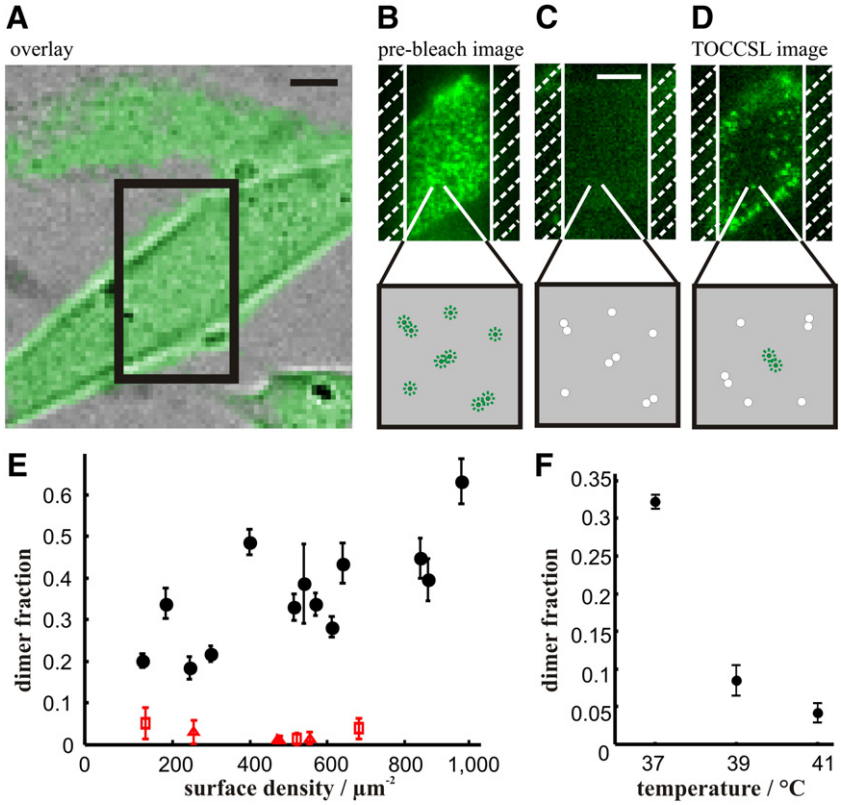

Fig. 1. Temperature elevation releases mGFP-GPI homo-association in the plasma membrane of living CHO cells. The principle of the TOCCSL method is shown in A–D. A, overlay of a white light image and the fluorescent image of a mGFP-GPI-expressing CHO cell. The box indicates the region of interest chosen for the TOCCSL sequence. B–D, different steps of the illumination protocol. The upper panels present the original data; the lower panels present a sketch of the effect on protein oligomers. Active and photobleached fluorophores are indicated by green and white dots, respectively. The white hatched areas indicate the position of the field stop (width of 7 µm). After recording the pre-bleach image (B), the selected area was totally photobleached by a laser pulse (450 ms). The efficiency of photobleaching was controlled by an image recorded immediately after the bleach pulse. After a recovery time of 800 ms, the first fluorescent spots entering the field of view could be observed as diffraction-limited signals. E, the dimer fraction as a function of the mGFP-GPI surface density at 37 °C. Each data point resulted from the analysis of a single cell. Experiments are shown under standard conditions (black circles) or after cholesterol depletion (red symbols). F, TOCCSL experiments were performed at indicated temperatures and dimer fractions were determined by pooling data from multiple cells. We found a substantial decline of the dimer fraction when increasing the temperature from 37 °C to 39 °C. At 41 °C mGFP-GPI homo-association was hardly detectable. Scale bars: 5 µm. "This research was originally published in [9]. © the American Society for Biochemistry and Molecular Biology."

that are transmitted to the central nervous system, leading to reflex or conscious homeostatic responses (reviewed by [40]). Some TRP channels, TRPV1, TRPV2, TRPV3, TRPV4 and TRPM3, are gated by warm temperatures, while others, TRPM8 and TRPA1, are gated by cooling. Collectively, TRP channels cover the entire range of physiological relevant temperatures, from the extreme cold to harmful heat stimuli. TRPV1 was the first mammalian TRP channel identified [41]. It is expressed in nociceptors and is gated by heat, low pH, capsaicin and endogenous lipids, playing a critical role in inflammatory pain and heat hyperalgesia [42]. Several recent reviews provide detailed information about the biophysical properties and physiological roles of TRP channels gated by temperature [35,40,43].

The molecular mechanism underlying temperature sensitivity of TRP channels is still unknown. The scarce structural information about these channels is currently a limiting factor in addressing this question. In the case of TRPV4, heat sensitivity appears to be mediated by the generation of an intracellular soluble ligand [44]. Also, it has been hypothesized that some TRP channels might sense temperature-mediated changes in lipid bilayer tension [36,39]. Other studies suggested that TRPV1 may be gated by heat indirectly, following the production of endogenous metabolites of linoleic acid [45]. However, purified TRPV1 channels reconstituted in artificial liposomes show intrinsic heat sensitivity that is unaffected by changes in bilayer tension [46]. Structural domains involved in heat sensing of TRPV1 are still uncertain but some results with chimeric channels implicate the intracellular C terminus [47]. In fact, Clapham and Miller postulate the participation of a large number of hydrophobic groups in the conformational changes taking place during temperature activation of TRP channels [48].

TRPM8 channels can be gated by cold temperature in artificial lipid bilayers, suggesting an intrinsic cold sensitivity [49]. A direct comparison of temperature sensitivity of TRPM8 channels expressed in recombinant systems with the sensitivity of the same proteins in native tissues has revealed important shifts in their absolute threshold [50]. These differences suggest that native channels may be modified by post-translational modifications (e.g. phosphorylation) or by interaction with auxiliary proteins. Very little is known about the interactome of specific TRP channels. Recent studies have identified proteins interacting with TRP channels and modulating their thermal sensitivity. These include Pirt, an integral membrane protein that modulates TRPV1 [51] and TRPM8 [52]. In the case of TRPM8, several post-translational modifications have been shown to modulate the activity of the channel. Thus, several studies indicate that TRPM8 is modified by N-glycosylation at a single residue near the extracellular mouth of the channel pore [53–55]. A detailed characterization of mutant channels, or wildtype channels treated with tunicamycin, showed that these channels had normal trafficking to the plasma membrane but that their temperature-dependent gating was impaired [54]. Recently, a covalent linkage of large polymeric chains of poly-(R)-3-hydroxybutyrate (PHB) to TRPM8 was reported [56]. TRPM8 mutants lacking specific PHB-binding sites, and wildtype channels treated with PhaZ7, a PHB depolymerase with serine hydrolase activity, showed strongly reduced activity to menthol or cooling. The authors postulated that the large PHB polymer subunits will stiffen or contract with low temperatures, leading to strong conformational rearrangement of the TRPM8 protein. The changes in PHB conformation could lead to alterations in the protein followed by channel opening. Serine residues in the S3–S4 extracellular linker are critical in supporting PHB interaction and gating.

3.1. Modulation of TRP channels by lipids

Lipids exert important modulatory roles on many classes of ion channels, and TRP channels in particular (reviewed by [57,58]). Lipid composition of neuronal membranes is varied and non-uniform. This heterogeneity, including lipids with varying degrees of unsaturation, different head group structures and different acyl chains is a complicating factor when analyzing the effects of specific lipids on TRP channels. In addition, some cellular membranes are strongly compartmentalized, facilitating rapid local variations in the concentration of specific lipids. Besides composition, other factors affect the physical properties of lipid membranes, most notably temperature (see Section 2). Thermodynamic changes in the lipid matrix surrounding the channels can also influence their gating behavior. The most direct way of assessing the effects of lipids on ion channels is to reconstitute purified proteins into membranes of defined composition, like artificial liposomes [46] of synthetic lipid bilayers [49,59]. Lipids known to modulate TRP channels include: membrane phosphoinositides, lysophospholipids and lysophosphatidic acid (LPA), cholesterol, linoleic acid, anandamide, lipoxygenase and cyclooxygenase metabolites of arachidonic acid (AA). Some active lipids are produced endogenously. In the next paragraphs we highlight the modulation of some thermally-sensitive TRP channels by lipids.

The importance of phosphatidylinositol 4,5-bisphosphate (PIP_2) in cell signaling derives from the fact that it is relatively abundant in the membrane and because it is the substrate for phospholipase C (PLC), leading to regulated fluctuations in the concentration of PIP_2 by activation of cell surface receptors [60]. Many TRP channels are sensitive to PIP_2 (reviewed by [58,61]). However, this modulation is complex and controversial in the case of specific channels (e.g. TRPV1). For most TRP channels (e.g. TRPM8 and TRPV5/6), gating depends on the presence of PIP_2 in the plasma membrane (reviewed by [62]). In the case of TRPM8, activity runs down in excised patches and recovers with perfusion of PIP_2 [49,63]. Similar results were obtained with channels reconstituted on lipid bilayers [59,64]. Other pharmacological or enzymatic manipulations of PIP_2 levels are consistent with an activating role on TRPM8 [65]. PIP_2 can also play a role in regulating the desensitization of the channel. Consistent with this scenario, PIP_2 depletion resulting of the activity of Gq/11-PLC-coupled receptors leads to inhibition of TRPM8, although one study indicates a direct inhibitory effect of Gα protein subunits on the channel [66].

The regulation of TRPV1 by PIP_2 appears to be more complex. Recently, Cao and colleagues investigated the modulation of purified TRPV1 by phosphoinositides in artificial liposomes. The authors convincingly showed that gating of TRPV1 by agonists, including temperature, does not require phosphoinositides [46]. Moreover, several phosphoinositides (PIP_2, PI4P) inhibited TRPV1 activity tonically. The interaction of TRPV1 with phosphoinositides was traced to the intracellular C terminus [46,67]. In contrast to these results, several other studies have shown that application of PIP_2 can activate TRPV1 channels in excised patches [68]. Finally, others have described dual effects of PIP_2 (partial inhibition and activation) depending on channel state [69]. Clearly, more studies are needed to sort out these differences.

Linoleic acid is an unsaturated omega-6 fatty acid with an 18 carbon chain, used in the synthesis of AA. Oxidized products of linoleic acid, produced endogenously during inflammation and tissue injury, are potent stimulators of TRPV1. In contrast, these products are inactive on TRPV2/3/4 [45].

Lysophospholipids, specifically lysophosphatidylcholine (LPC) and lysophosphatidylserine (LPS), activate TRPM8 and sensitize responses to cold temperature [70,71]. These lipids are generated by calcium-independent PLA2 activity. These lysophospholipids are conically shaped, changing membrane curvature when inserted asymmetrically. On the basis of interactions between LPC and cationic amphipaths (e.g. tetracaine and chlorpromazine), the authors postulated local changes in bilayer tension as determinants of lysophospholipid effects on TRPM8. Similarly, other compounds partitioning in the lipid bilayer, such as volatile anesthetics (e.g. halothane, chloroform), also modulate TRPM8 activity [72]. Interestingly, LPC does not activate TRPV1 [73]. In contrast, LPA, an enzymatic product of lysophospholipids, produced in great amounts during tissue injury, activates TRPV1 channels directly by binding to a positively charged amino acid (Lys710) on the C terminus [73]. LPA was ineffective on TRPV2/3 and TRPA1. Because the

actions of extracellularly applied LPA were rapid, the authors postulated an efficient transbilayer movement of the molecule.

The endocannabinoids *N*-arachidonoyl-ethanolamide (anandamide) and *N*-arachidonoyl-dopamine (NADA) [57,74,75] activate TRPV1. Anandamide binds to TRPV1 with relatively low affinity and potency. In contrast to the activating effects on TRPV1, anandamide and NADA inhibit TRPM8 activity [75]. Consistent with this finding, several plant-derived cannabinoids including cannabidiol, cannabidiol acid, cannabigerol, Δ9-tetrahydrocannabinol, and Δ9-tetrahydrocannabinol acid, also inhibit menthol- and icilin-evoked Ca^{2+} increases in TRPM8 expressing cells with IC_{50}-values in the submicromolar range [75].

Other endogenous lipids activating TRPV1 include lipoxygenase metabolites of AA, in particular 12-(S)-hydroperoxyeicosatetraenoyl acid (12-(S)-HPETE), 5-(S)-hydroxyeicosatetraenoyl acid (5-(S)HETE), and leukotriene B4 (LTB4) [76]. Farnesyl pyrophosphate, an isoprene lipid in the cholesterol synthesis pathway, activates TRPV3 [77] without effects on TRPV1 [73].

Cholesterol is an essential lipid in the plasma membrane of eukaryotic cells. Lipid-rafts are lateral membrane microdomains enriched in cholesterol and sphingolipids [18,78]. The activity of some TRP channels (e.g. TRPV1 and TRPC3) is sensitive to membrane cholesterol content, suggesting that raft association is pivotal for the physiological role of these channels. In addition, lipid rafts can compartmentalize ion channels and other signaling proteins [79]. Several TRP channels have been shown to segregate into cholesterol-rich lipid microdomains including TRPC1, TRPC3, TRPC4 and TRPC5 and TRPM8 [80–82]. In the case of TRPM8, lipid-raft disruption with methyl-β-cyclodextrin (MBCD) increased both menthol- and cold-mediated activation. Moreover, the threshold to activate TRPM8 shifted to higher temperatures when the channels were displaced outside of lipid-rafts [81,83]. These results indicate that the lipid-membrane environment modulates the properties of TRPM8 channel as a cold sensor and suggests a role for lipid-rafts in cold transduction.

Recently, an essential contribution of TRPV1 was demonstrated in heat-inducible Hsp induction [84]. As such, it is tempting to speculate that TRPV receptors might form a new class of therapeutical targets enabling us to modulate the HSR response. For example, by inhibiting their activity, an overactive HSR – as seen in multiple cancers – might be tempered subsequently potentiating the effect of various chemotherapeutical strategies.

Taken together, lipids modulate the activity of thermally-gated TRP channels by multiple mechanisms. In some cases, lipids exert direct effects on the channel, acting as allosteric modulators that modify the sensitivity to other agonists (temperature, chemical agonists). In other cases, the effects of lipids appear to be indirect, altering the mechanical properties of the membrane, leading to gating of the channel. From the functional point of view, direct and indirect effects are equally relevant in shaping the response of TRP channels to environmental stimuli.

4. Stress hormones and the membrane-regulated stress response: heat shock can alter membranes indirectly through the elevation of plasma glucocorticoid levels

A coordinated neuroendocrine response is mounted following exposure to a variety of environmental stressors, including temperature changes, in order to minimize homeostatic disturbances [85]. The physiological response to acute stress is relatively well conserved among vertebrates and involves the rapid release of stress hormones, primarily catecholamines and glucocorticoids. Once stress hormones are secreted into the blood, they act on target tissues to stimulate physiological adjustments that allow the organism to cope with stress [86]. In addition to receptor-mediated responses, stress hormones also interact with plasma membrane lipid and protein components, resulting in varying degrees of perturbations to the physicochemical properties of the plasma membrane [87]. Since glucocorticoids diffuse through the plasma membrane, biophysical alterations induced by this steroid are of particular interest. Moreover, glucocorticoids are traditionally associated with eliciting delayed effects by activating the glucocorticoid receptor (GR) and the associated genomic signaling events; however, they also elicit rapid cellular responses that are nongenomic [88]. Although seldom investigated, the possibility exists that biophysical changes to the plasma membrane are integral to non-genomic actions of steroids [87].

Ectothermic organisms, in particular, are exposed to a greater range of environmental disturbances and typically possess enhanced homeostatic defenses [89] making them ideal organisms to study membrane responses. Recently it was demonstrated that stressor-mediated elevation in corticosteroid levels alter the fluidity of liver plasma membrane, potentially leading to modulation of signaling pathways in rainbow trout [90,91]. This finding implicates the plasma membrane as a potential regulator of stress hormone-mediated cellular effects and indicates that stressors, including heat shock, can alter membranes by directly altering intermolecular interactions, as well as indirectly through the elevation of plasma glucocorticoid levels. In rainbow trout (*Oncorhynchus mykiss*), cortisol significantly fluidizes hepatic plasma membrane in vitro at concentrations comparable to endogenous stress levels (100–1000 ng/ml) [91]. The fluidity changes seen with cortisol were not dose-dependent, but instead occur at concentrations ≥100 ng/ml, which are typical levels observed in stressed salmonids [91]. The all-or-none response coupled with the inability of an impermeable cortisol–peptide conjugate to alter lipid order suggests a receptor-independent mechanism that depends on steroid partitioning into the lipid bilayer [91]. In rat erythrocytes, corticosteroids were shown to have a greater impact on membrane order near lipid–protein domains compared to regions devoid of protein [92]. This underscores that microviscosity alterations due to cortisol incorporation into lipid bilayer may play a fundamental role in regulating signal transduction pathways, including stress-activated cascades.

The fluidity of the lipid environment also affects lipid raft formation and stability [93]. Indeed, atomic force microscopy imaging, which measures surface properties of membranes, has revealed that cortisol alters structural organization of membrane microdomains in rainbow trout [91]. The physical perturbations mediated by cortisol are not uniform between microdomains, as cortisol altered the surface adhesion of lower membrane domains, but had a minimal effect on higher membrane domains [91]. Membrane domain reorganization in response to cortisol was also reported in rat erythrocytes and involved formation of large protein–lipid domains and changes in membrane elasticity [92]. Other stress hormones, including epinephrine and norepinephrine, also altered erythrocyte plasma membrane topography, further suggesting that physical changes to the plasma membrane that are hormonally-mediated may be important for mounting a cellular response to stressors. More importantly, while stress hormones increased membrane microviscosity of rat erythrocytes, each had a distinct effect on microdomain formation suggesting that biophysical alterations are hormone-specific [92], and changes to membrane organization may play an important role by which hormones coordinate and integrate cellular stress response.

Activation of the systemic physiological stress response and its effect on biophysical alterations to the plasma membrane were recently assessed in rainbow trout liver [90]. The experiment consisted of a brief handling disturbance of rainbow trout injected with either saline (sham) or metyrapone, an 11β-hydroxylase inhibitor that blocks endogenous cortisol biosynthesis [94]. Interestingly, this study demonstrated that a physical stressor eliciting a cortisol response was sufficient to rapidly fluidize liver plasma membranes, and this was further confirmed by the absence of a stressor-effect on membrane properties in the metyrapone group (Fig. 2). Changes in lipid order were accompanied by an increase in membrane roughness, similar to those seen with cortisol treatment in vitro [90]. This novel finding suggests that cortisol incorporation into the lipid bilayer increases membrane fluidity in vivo, while the mechanisms remain to be elucidated [90]. Stressor-mediated microdomain reorganization likely has important

functional consequences as alteration in lipid rafts plays a critical role in regulating receptor activity and intracellular signaling [78]. Moreover, raft-dependent signaling cascades are reportedly more sensitive to membrane disturbances in the liver since hepatocytes lack caveolae, which are stable membrane domains that are enriched with oligomeric complexes of caveolin [95].

Stress hormones are also established regulators of the HSR [96]. In response to thermal stress, cortisol facilitates mobilization of energy stores to meet the enhanced metabolic demands during thermal disturbances. In addition to metabolic reprogramming, cortisol also regulates the cellular stress response by attenuating the production of Hsps, which likely helps to restrict excessive use of cellular energy needed for de novo protein synthesis [97]. Currently, the mechanism by which cortisol attenuates the HSR in teleosts is poorly understood but seems to involve GR degradation by the proteasome [98]. In rainbow trout cortisol fluidizes liver plasma membranes [90,91], therefore, one might expect that cortisol would enhance, rather than attenuate, Hsp induction. However, not all fluidizing agents activate the HSR, and instead the nanomolecular perturbations to microdomain structure likely determine the intracellular response [99]. Cortisol-induced biophysical alterations are also accompanied by rapid and transient activation of stress-activated kinase pathways, including ERK1/2, AKT, PKC and PKA (Fig. 3; [90,91]). These stress-activated kinase pathways are also known to respond to membrane perturbations (see further) [100,101], including biophysical alterations mediated by benzyl alcohol (BA) in rainbow trout liver [91]. Moreover, it has been demonstrated that phosphorylation by ERK1/2 and PKC leads to the suppression of HSF1 activity in humans [102–104]. Although the role of phosphorylation in regulating teleost HSF1 transcriptional activity is currently unknown, we propose that the regulation of stress-activated kinases by cortisol-mediated membrane perturbations may also play a role in the attenuation of the HSR in fish.

Combined, there is mounting evidence for a novel membrane-mediated stress adaptation response through direct perturbations to the plasma membrane in response to stress hormones. The interaction of stress hormones with the plasma membrane significantly impacts membrane fluidity and stimulates membrane microdomain restructuring. The cortisol-mediated membrane restructuring is associated with enhanced phosphorylation of stress-activated proteins, underscoring a functional role for this steroid in rapid stress signaling and the overall cellular stress response.

5. Mimicking the effect of high temperature and the mode of action of membrane-perturbing Hsp inducers by computational techniques

Once the physical state of a membrane is modified, the membrane permeability and/or the membrane protein activity may change. The

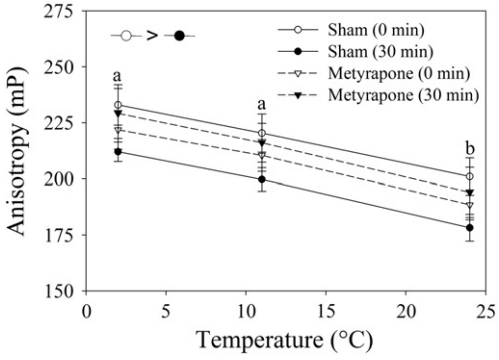

Fig. 2. Effect of acute stress on 1,6-diphenyl-1,3,5-hexatriene (DPH) fluorescence anisotropy. DPH fluorescence anisotropy (inversely related to membrane fluidity) was measured in enriched hepatic plasma membranes isolated from sham or metyrapone-treated trout before and 30 min after a handling stress. Data represents mean ± S.E.M. (n = 4–6). Different upper case letters and inset indicate significant time and treatment effects, respectively (two-way ANOVA, $p < 0.05$). This figure was originally published in [90].

complex interplay between chemical–physical membrane state and cell membrane activities continues to be object of investigation. Zhang et al. [105] and Rossignol et al. [106] studied the influence of membrane fluidity on the lysosomal permeability to H^+ by fluidizing and rigidifying the membranes with BA and cholesteryl hemisuccinate, respectively. They demonstrated that the increases or decreases in membrane fluidity correspond to the increases or decreases in their proton permeability. Deamer and Nichols [107] found that the proton permeation correlates with the physical properties of the membranes and involves transient formation of hydrogen bonded chains of water within the membranes.

A small fraction of the water in the bilayer might be associated through hydrogen bonding, thereby providing a proton conductive pathway. The permeation of protons across membranes is suggested as being along the hydrogen bonds of water, which crosses the membranes through aqueous pores [108] and transient defects [109]. It has been established that fluidizing or rigidifying membranes can increase or decrease the size of their aqueous pores, respectively [110], and that the transient defects of membranes can be increased in number by the temperature-dependent phase transitions or by membrane fluidizers. It should be noted that a number of studies find little correlation between membrane fluidity and proton permeability for some membranes [111,112]. These studies suggested that the effects of membrane fluidity and water permeability on the proton permeation are dependent on the membrane types.

Recently, we [113] demonstrated a direct correlation between the membrane fluidization of the lipid region and the Hsp response for mammalian cells. We chose BA and heptanol as membrane fluidizers in model membranes to follow the idea that weakening the interactions between lipid chains could induce a disordering effect in membranes similar to the heat shock. The idea that the modification of the lateral lipid organization is responsible for membrane dysfunction is particularly attractive in the context of the lipid raft model.

In mammalian cells, a thermal shift up to 42 °C triggers the HSR, accompanied by increases in the expression of genes for stress proteins such as Hsp70. Similarly, the addition of small molecules capable to act as membrane fluidizers can induce a similar response [113]. The most critical aspect of the term “fluidity” is that it is not operationally well defined, since a bilayer is inherently anisotropic and the effect of different fluidizers can be observed in different portions of the membrane [114].

Molecular dynamics (MD) is a computational technique that permits to gain insight in the lipid environment without the use of bilayer-perturbing probes. Unfortunately, until very recently, lipid molecules were not well parameterized and the time length of the simulations, usually less than 10 ns, precludes the observation of important phenomena such as water permeation and raft evolution. In recent years, several MD studies have been published to verify the effect of chemical fluidizers in membranes [8,115–118].

Here, we report a molecular dynamics study of 3 small molecule heat shock protein inducers (BA, phenethyl alcohol (PhA), and bimoclomol (BM)) all capable to perturbate lipid membranes similarly to thermal stress [113,119]. The goal was to correlate their documented effects on the HSR with the differences in the interaction of these molecules with 4 model membranes designed by computational modeling.

Four model membranes have been used as controls: two membranes are made of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), respectively. A third membrane was a mixture of POPC and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) with molar ratio 1:1. The fourth membrane is made of sphingomyelin (SM) and cholesterol (CHOL) in molar ratio 1:1. The first three mixtures (POPC, DMPC, POPE) model membranes in the Ld phase, while the fourth mixture (SM/CHOL) represents lipid rafts in the Lo phase (see Box 1).

5.1. Effects of membrane perturber Hsp inducers: benzyl alcohol, phenethyl alcohol and bimoclomol

In order to determine the mechanisms followed by BA, PhA and BM administration to modulate membrane nanostructure and to compare the differences obtained in respect of the thermal effect, we have performed a molecular dynamics investigation of the three molecules incorporated in 4 membranes of different intrinsic fluidity: POPC/POPE, POPC, DMPC and SM/CHOL.

In Table 1 we indicated two of the most important structural parameters: surface area per lipid and thickness of the bilayer. Within the approximation of the AMBER03 force field, we obtained stable membranes with values slightly smaller than those derived from experimental observations. Upon addition of the Hsp inducers we have calculated the total potential energies after an equilibration run of 20 ns. The energies have been normalized on the molecular weight to permit appropriate comparison. The main results are shown in Table 2.

As a common feature, BA, PhA and BM molecules show a clear ability to penetrate the membrane. The distribution coefficient at pH 7.4 of PhA and BA is higher than the one of BM, indicating that the two alcohols are completely absorbed in lipid membranes, whereas BM distributes between lipid membranes and water. Even if the molecules are initially placed in the bulk aqueous phase, the membranes quickly absorb BA and PhA. BM appears to be equally partitioned between water and membranes.

Interestingly, BA and PhA stabilize the SM/CHOL bilayer, but increase the energies in POPE/POPC, POPC and DMPC membranes. BM acts in an opposite manner: it causes stabilization of Ld membranes and remains almost ineffective in Lo membranes. Apparently, BA and PhA, less bulky, can better interact with SM molecules in SM/CHOL membranes. Though BA and PhA span the inner core of the membranes, they prefer to expose the hydroxyl group to the membrane/water interface. Within the approximation of the AMBER force field, BA and PhA show the same potential energy variations in all tested membranes.

With an isoelectric point of 11.56, BM is positively charged at physiological pH and is completely absorbed on negatively charged membranes containing phosphoserine. Nevertheless, BM is also capable of interacting with neutral membranes, preferring more fluid membranes, whereas BA and PhA accumulate in cholesterol-containing bilayers, with higher spacing among headgroups. The difference in potential energy depends on the balance between the dipole–dipole interactions between compounds and membrane headgroups and in the packing change of the lipids. Besides the internal ordering and conformation of the lipid components, it is important to characterize a membrane in terms of its lipid lateral organization. In the case of the POPC, the lateral spacing between hydrophilic headgroups is higher because of the acyl chain cis-double bond, which leads to increased hydration. Consequently, the lateral packing is weaker and the insertion of more bulky BM is easier. In SM/CHOL membranes, cholesterol was found to increase the number of holes in the polar region of bilayers and thus to lower the solvation free energy for small molecules in this region. BM tends to

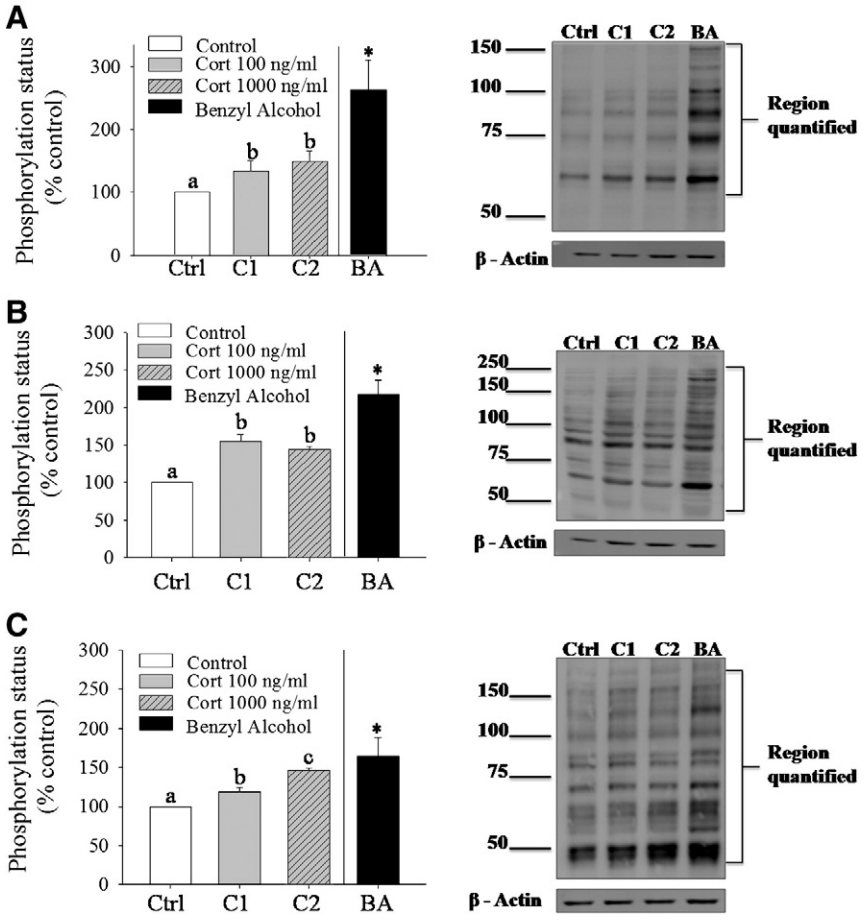

Fig. 3. Cortisol effect on rapid cell signaling in trout hepatocytes. Rainbow trout hepatocytes were incubated either with cortisol (0, 100 or 1000 ng/ml) or BA (25 mM) for 10 min. Cell homogenates (40 μg protein) were probed with polyclonal rabbit antibody (Cell Signaling Technology, Beverly, MA) to either phospho-(Ser) PKC substrate (A), phospho-PKA Substrate (RRXS/T) (B) or phospho-AKT substrate (RXXS/T) (C). Equal loading was confirmed with β-actin (monoclonal mouse antibody; Sigma, St. Louis, MO). A representative immunoblot for each is shown; values are plotted as % control and shown as mean ± S.E.M. (n = 3 independent fish); bars with different letters are significantly different (repeated measures ANOVA, $p < 0.05$). *Significantly different from control (Paired Student's t-test; $p < 0.05$). This figure was originally published in [91].

localize behind the phosphorous atoms of the bilayers and it affects the cholesterol alignment and the headgroup orientation. BA, PhA and, more intensively, BM cause lateral disorder in lipid bilayers. Lo phases display a certain degree of periodicity of their lipids in the plane of the bilayer, whereas in Ld phases such organization is lost at very short distances. The lateral order in membranes was quantified by calculating the pair density correlation functions (data not reported).

DMPC lipid acyl chains become less ordered in the presence of BA and PhA. Since POPC and POPC/POPE membranes are already in the fluid phase at room temperature, the fluidity changes are much smaller than that observed in DMPC membranes. BA and PhA induced a disordering effect by weakening the van der Waals interactions between the lipid acyl chains. BM has a stronger effect on the lateral stress profile (data not reported) and therefore it can modify the protein helix–helix interaction along with the overall fluidity.

Mass density profiles provide information on the averaged displacement of membrane lipids upon the insertion of an exogenous molecule. In Fig. 4 the density profile of the Lo membrane SM/CHOL is indicated. Fig. 4A shows the atom distribution of the pure membrane. This distribution, with cholesterols toward the membrane surfaces and with the hydroxyl group under the SM headgroups (the so called umbrella model) is clearly modified upon addition of BA (Fig. 4B), PhA (Fig. 4C), and especially of BM (Fig. 4D). BM binding to the membranes is capable to cause the sinking of the cholesterol toward the middle of the membranes, increasing the mobility of the hydrophobic regions immediately under the SM headgroups. These results show that BM reduces the mobility of SM headgroups and the mobility of terminal carbons, but due to its effect to displace cholesterol, it may increase the mobility of the chains toward the middle of the bilayer.

In conclusion, the mechanism of action of BA and PhA is typical of anesthetics, in which BA and PhA replace water molecules that normally penetrate under the phosphorus atom level, decreasing lipid chain repulsion and the order parameters. Contrarily, BM, which bears a positive charge at pH 7.4, preferentially interacts with negatively charged membranes. With neutral membranes, BM can penetrate slightly under the phosphorous levels and it is capable to stabilize membranes containing nonbilayer forming lipids (Table 1).

Box 1
The design of model membranes.

The POPC membrane consisted in 98 POPC molecules; the simulation box dimensions were X = 120.0 Å, Y = 57.18 Å and Z = 56.42 Å filled with 24 Cl^- and Na^+ counter ions and 8265 water molecules. The POPC/POPE (60–40%) membrane consisted in 58 POPC and 40 POPE molecules; the simulation box dimensions were X = 109.4 Å, Y = 56.69 Å and Z = 53.79 Å filled with 20 Cl^- and Na^+ counter ions and 7098 water molecules. The SM/CHOL membrane is made of 135 SM and 137 CHOL molecules. The initial box dimensions were X = 73.79, Y = 104.61, Z = 72.62, filled with 26 Na^+ and Cl^- counter ions and 10,498 water molecules. The DMPC membrane is made of 72 DMPC molecules. The initial box dimensions were X = 99.60, Y = 42.46, Z = 42.33, filled with 3590 water molecules. The coordinates have been taken from a previous work on phosphatidylcholine (PC) membrane [120]. The volumes of BA, PhA and BM are of 101.40 $Å^3$, 116.22 $Å^3$, and 264.43 $Å^3$, respectively. Upon insertion in the bilayers, the membranes have been further relaxed with a steepest descend followed by a simulated annealing until the maximum atomic speed was slower than 500 m/s. All the simulations were performed with the program YASARA [121] under NPT ensemble at 310 K and 1 atm by coupling the system with a Berendsen thermostat [122] and by controlling the pressure in the manometer pressure control mode. The force field utilized was the AMBER03 [123]. The geometry of the molecules was optimized by semi-empirical AM1 method using the COSMO solvation model [124]. Partial atomic charges were calculated using the same level of theory by the Mulliken point charge approach [125]. Electrostatic interactions were calculated with a cutoff of 10.48 Å, and the long-range electrostatic interactions were handled by the Particle Mesh Ewald (PME) [126] algorithm using a sixth-order B-spline interpolation and a grid spacing of 1 Å. The leap-frog algorithm was used in all simulations with a 1.25 fs time step for intramolecular forces and 2.5 fs time step for intermolecular forces and the equilibration period was of 2 ns. The lipid bilayers were assembled and relaxed reducing the box dimension till the van der Waals energy of the system starts to increase [120,127] and the structural parameters of the membranes are comparable to previously published experimental data [23]. To remove bumps and correct the covalent geometry, all the systems were energy-minimized. After removal of conformational stress by a short steepest descent minimization, the procedure continued by simulated annealing (time step 2 fs, atom velocities scaled down by 0.9 every 10 steps) until convergence was reached, i.e. the energy improved by less than 0.05 kJ/mol per atom during 200 steps. A full description of the protocols is given in [8,127].

6. Cell profiling and lipidomics: novel tools to explore the primary membrane-associated events critical in the triggering of stress signals

The study of the cellular stress response is of great importance to our understanding of how cells respond and adapt to various changes in their environments especially during different pathophysiological conditions. Fundamental questions relate to whether all cells in a culture (or in tissue) suffer the same consequence of an imposed stress and whether they respond in the same manner. Knowledge about gene expression and cellular responses in cells is derived from analyses of populations consisting of millions of cells. Although this approach provides useful insights into average population responses, they do not provide information on individual cells or subpopulations within cell cultures. Data obtained by common methods can be misleading, especially if the responses of cells are heterogeneous or if subpopulations exist. Thus, using the classical biochemical, biophysical approaches, we are potentially missing a large amount of data on cells in populations. For this reason, it is necessary to record data from many individual cells so as to acquire a statistically significant sampling of cellular behavior and dynamics. One of the most common techniques for this task is flow cytometry which allows multiple parameters on individual cells to be obtained and these cells could also be sorted for further study [128]. Flow cytometry, on the other hand, cannot follow the same individual cells in time. This quest depends heavily on molecular imaging, which shows when and where genetically or biochemically defined molecules, signals or processes appear, interact and disappear, in time and space. With high-content cellular analysis, it is necessary to perform high-throughput phenotype profiling, linking gene expression to biochemical signaling pathways in the cell and, ultimately, to cell behavior. By combining state-of-the-art ultrasensitive imaging techniques, flow cytometry and biochemical analysis we can gain insight into the heterogeneous cellular stress response.

6.1. Background of cell population heterogeneity

Cells within isogenic populations exhibit broad phenotypic variation. For experimental purposes, such variation is readily apparent in phenotypes that are selectable. Almost any study that has used measurements of % viability (or other similar parameter) and documented an intermediate value (i.e. other than 0% or 100%) is indicative of cell-to-cell variation. This nongenetic heterogeneity can be fundamental to

Table 1
Comparison of structural parameters derived from experimental observations and molecular dynamics simulations after 50 ns.

Membrane composition	Surface area per lipid (Å^2)		Average thickness of the lipid bilayer (Å)	
	Experimental	Molecular dynamics	Experimental	Molecular dynamics
DMPC	61.7	61.1	35.7	33.1
POPC	69.5	68.9	39	35.3
POPE/POPC (50:50)	56.6–69.5	56.5–68.9	40	36.5
SM/CHOL (50:50)	49.5–29.6	49.2–28.3	42	38.8

Standard deviations were 0.5 Å^2 and 0.3 Å^2 for surface area per lipid and average thickness of the bilayer, respectively.
Experimental values are from: http://www.brocku.ca/researchers/peter_rand/lipid/default.html.

the fitness of an organism, particularly where there is little genetic heterogeneity. The term persistence is used to describe the minority of cells within a genetically homogeneous microbial population that is able to survive a period of antibiotic treatment. Phenotypic persister cells were first reported over 60 years ago. In 1944, Joseph Bigger noticed that the newly-introduced penicillin was unable to "sterilize" a culture of *Staphylococcus* [129]. His experiments indicated that persisters were not mutants, and upon reinoculation produced a population containing a sensitive bulk and new tolerant cells. The result clearly showed that surviving persisters regenerate the original population, and are therefore phenotypic variants of the wild type. Similar phenomena have now been documented in microorganisms exposed to a wide range of antibiotics and other toxic agents (reviewed by [130]).

While microbial heterogeneity is well documented in both natural and laboratory environments, the underlying causes are less well understood. The cell cycle, cell aging and epigenetic regulation are proven drivers of heterogeneity in several of the best-known phenotypic examples. However, the full contribution of factors such as stochastic gene expression is yet to be realized.

The available cell population heterogeneity studies in mammalian cells are limited. A cell-to-cell variability in lipid droplet formation was described as depending on the cascade responses of an insulin signaling pathway which includes insulin sensitivity, kinase activity, glucose import, expression of an insulin degradation enzyme, and insulin degradation rate. Increased and prolonged insulin stimulation promotes lipid droplet accumulation in all differentiating cells. Single-cell profiling revealed the kinetics of an insulin signaling cascade as the origin of phenotypic variability in drug-inducible adipogenesis [131]. A recent study of transcription activation in a serum starvation assay highlighted the wide variety of responses among individual cells, even though the large differences between the control and treatment groups were scored in a population assay such as an immunoblot [132].

6.2. Heterogeneous cellular stress response

Heterogeneous stress resistance is widely documented in reports dealing with virtually any stressor–microorganism combination. Such heterogeneous resistance can be confirmed to be non-inheritable (that is, non-genotypic), as has been shown for acid-stress and osmotic-stress resistance in *Escherichia coli* [133]; and for sorbic-acid [134] and heat resistance [135] in *Saccharomyces cerevisiae*. Analysis of stress responses in single yeast cells revealed a remarkable variability among individual cells in clonal populations (reviewed by [136]). Although mean gene expression was affected by genetic background, the heterogeneity of gene expression among individual cells in clonal populations was described to reflect physiological differences between cells like different stages of cell cycle or replication, or exposure to different local microenvironments.

The chaperone Hsp90 is reported to also function as an 'evolutionary capacitor', suppressing the expression of genotypic variation in a population [137]. It is proposed that the susceptibility of this capacitor function to environmental perturbation serves to promote genotypic heterogeneity when it is most likely to be advantageous to the organism. This premise has been extended to non-genotypic heterogeneity. From modeling studies with yeast, it was suggested that a multitude of cellular gene products might function like capacitors by buffering variable expression of other genes in gene-regulatory networks [138] In addition to the above roles, Hsps can influence cellular stress resistance directly. Evidence for single-cell variability in this regard has come from in situ studies using RT-PCR in *Salmonella enterica* serovar Typhimurium, in which the levels of mRNA for the Hsp GroEL displayed significant heterogeneity between individual cells [139]. It was suggested that this heterogeneity was related to the variability in the cell-cycle position of individual cells in growing cultures. Cell-cycle dependent heat resistance has been reported in *S. cerevisiae* [140]. Further work revealed that individual cells in *S. cerevisiae* subpopulations exhibited a 1500-fold variation in the induction of the *Hsp104* promoter by mild heat shock [135]. The strength of the *Hsp104* promoter induction correlated with the cell-to-cell variation in resistance to a lethal heat stress, establishing a phenotypic consequence for the heterogeneous transcriptional response. The mechanistic basis for this heterogeneous response is not genotypic (the phenotypes were not inherited); however, the role of the cell cycle or other parameters that could function as the underlying driver of this heterogeneity was not investigated. One interesting observation that could be relevant is the short-period (~3–6 min) shuttling to and from the nucleus exhibited by the

Table 2
Effect of membrane perturber Hsp inducers on the total potential energies of different membrane models.

Membranes	Membrane potential energies (kcal/mol)				Percentage changes compared to the pure membranes		
	Pure	BA	PhA	BM	BA	PhA	BM
POPE/POPC	8704 (119)	9076 (137)	9070 (129)	7101 (121)	+4.3%	+4.3%	−18.4%
POPC	11,216 (121)	11,547 (137)	11,560 (140)	11,675 (116)	+2.9%	+3%	+4%
DMPC	9086 (148)	9458 (129)	9505 (130)	8049 (132)	+4.1%	+4.6%	−11.5%
SM/CHOL	16,827 (125)	16,234 (134)	16,237 (138)	17,110 (134)	−3.5%	−3.5%	+1.7%

Energies are calculated on the basis of AMBER03 force field. The values have been normalized on the molecular weight of the system (bilayer + solvent + fluidizers). The error (within brackets) is the standard deviation of energy values calculated every 0.5 ns in the last 5 ns of simulations.

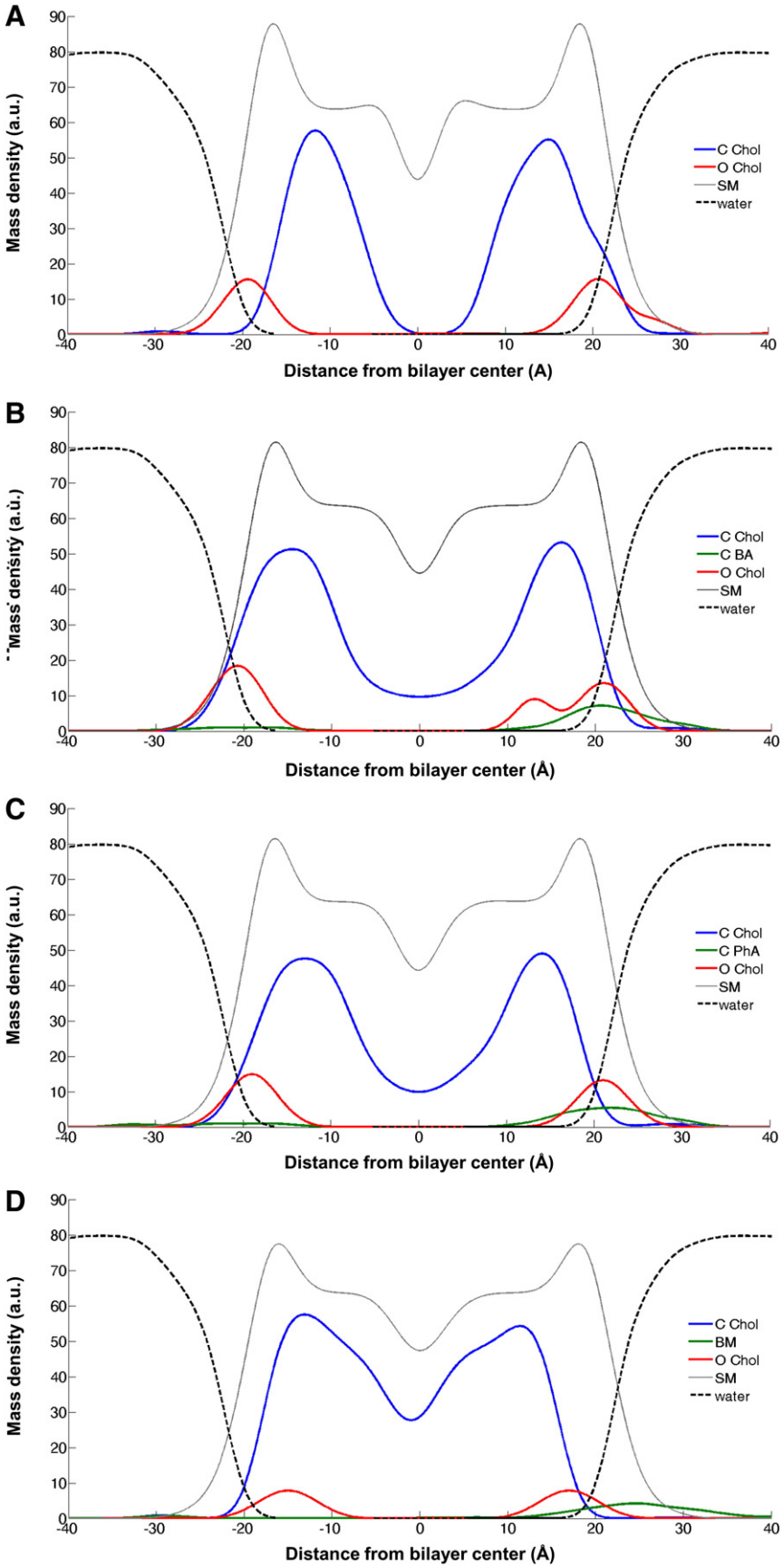

Fig. 4. Mass density profiles of SM/CHOL (135:137) membrane. The atom distribution of the pure membrane (A); upon the addition of BA (B), PhA (C), and BM (D). The dashed black line represents water molecules, the thin gray line is the overall density of SM. Cholesterol oxygens and carbon atoms are shown in red and blue, respectively. BA, PhA and BM are indicated in green.

transcriptional activators (Msn2p and Msn4p) of the general stress response in *S. cerevisiae* during adaptation to mild stress [141]. Superimposed on this cyclical behavior is the marked heterogeneity between individual cells in the patterns and timing of Msn2p and Msn4p shuttling. In continuous yeast cultures, nuclear localization of the redox-sensitive transcription factor Yap1p is also known to oscillate [142]. The respiratory oscillations in yeast – often termed ultradian rhythms – could underlie phenotypic heterogeneity in a broad range of resistance phenotypes. Linked to these oscillations, resistance to the pro-oxidants hydrogen peroxide (H_2O_2) and menadione, as well as to heat, cycled in phase with each other; that is, resistances to these different stressors reached maxima or minima at the same times during the oscillation [143,144].

Resistance to an 'uncoupler' of mitochondria and to the toxic metal cadmium also oscillated, but these were slightly out of phase with the other stressors. Some preliminary evidence indicates that redox cycling of antioxidants, such as glutathione, during respiratory oscillation could underpin at least some of the above phenotypes [130]. Ultradian oscillations are typically believed to be linked to the cell division cycle. Direct associations between cell-cycle position and pro-oxidant resistance have been demonstrated; the G1 and G2 stages of the *S. cerevisiae* cell cycle were identified as menadione-resistant and H_2O_2-resistant phases, respectively [145].

Microvascular endothelial cells were shown to respond heterogeneously to shear stress. Data suggested that shear stress stimulates cells to release ATP, causing the increase in intracellular calcium concentration via purinergic receptors in cells that are heterogeneously sensitive to ATP. By this means, ATP acts as an autocrine and paracrine hormone, serving to integrate the heterogeneity of individual cell responses and coordinate vascular functions [146].

6.3. Cell to cell variation of membrane heterogeneity

In recent years, our understanding of the plasma membrane has changed considerably as our knowledge of lipid microdomains has expanded. These include structures known as lipid rafts and caveolae, which are readily identified by their unique lipid constituents. Cholesterol, sphingolipids and specific phospholipids with more saturated fatty acyl chain moieties are typically highly enriched in these lipid microdomains. Since lipid microdomains have been widely shown to play important roles in the compartmentalization, modulation and integration of cell signaling, we suggest that these microdomains may additionally have an influential role in stress sensing and signaling. Since the raft structure is also dependent on the thermally controlled lipid-phase behavior, even mild changes in temperature could result in a fundamentally altered solubility and consequently redistribution of these proteins in the rafts. More severe heat may result in complete raft dissolution and disruption of the signaling activities [147].

The available literature data about cell population heterogeneity of the heterogeneous membrane structure is very limited. A higher heterogeneity of plasma membrane in multidrug-resistant LR73 carcinoma cells was found in comparison with non-resistant cells [148]. A reduced degree of heterogeneity was found upon treatment with the membrane fluidizing agent BA. A relation between the distribution of the diffusion-time values and the modification of membrane lateral heterogeneities was proposed [148].

Our pilot studies on the heat shock response of B16 melanoma cells showed a significant variation of both membrane domain organization and the expression level of Hsp70 promoter driven YFP (Peksel et al., unpublished).

6.4. "Zooming in" on membrane hyperstructures engaged in the generation of stress signal

During or after the imposition of stress on cells we can zoom in on individual cells or individual fluorescently tagged molecules, using an ultrasensitive, high-speed camera attached to a fluorescence microscope in TIRF mode to observe what happens in the membranes as each cell reacts to the particular treatment. A change may occur in the topology of a particular membrane domain, a candidate from which an initial heat shock signal could originate, or a membrane receptor may be activated, allowing the monitoring in real time as a receptor complex responds and activates signaling pathways [99]. An intracellular signaling molecule tagged with a fluorescent protein could light up or could form a FRET pair as it interacts with another component of the signaling pathway, perhaps relaying instructions to the nucleus to activate or deactivate a particular stress defensive gene. The particularly valuable aspect of this methodology is not the astounding visual images it produces, but rather the abundant and diverse data that can be extracted from those images—data that afford a better understanding of what is happening in the cell in response to stress. Image analysis of this nature requires efficient imaging algorithms, such as the open source Cell Profiler (www.cellprofiler.org), which provides a selection of tools for automatic analysis of the cell morphology, and the localization and intensity distribution of fluorescent markers [149]. The availability of high content imaging tools allows us to link gene expression to biochemical signaling pathways in individual cells and, ultimately, to cell behavior. During or after the imposition of stresses on cells we can zoom in on individual cells using ultrasensitive, high-content, time-lapse fluorescent microscopy with a very high spatial and temporal resolution to observe what happens as each cell reacts to the given treatment. We developed robust tools for automatic image analysis to follow the individuality of dynamic cellular events in a population of cells during the development of the stress response. The results gained with our cell profiling approach revealed specific, heat induced membrane rearrangements (Peksel et al., unpublished). The ability of cells to respond to stress correlated with the structure of the plasma membrane prior the heat treatment. By changing this specific membrane structure by chemical compounds the HSR could be altered and a selective remodeling of the HSR could be achieved [4].

6.5. Lipidomics of the heat shock response

It is well established that stressful conditions activate numerous membrane-related sensors at the top of the signaling pathways which are interconnected by the parameters of the membrane chemical and physical state. Phospholipids, sphingolipids and cholesterol are also involved in stress-induced second messenger generation [4,150]. A precise identification and quantitation of the cellular lipids is therefore a prerequisite to map the interactions and dynamics of lipids during stress response. The recent advances in mass spectrometric techniques facilitated the development of lipidomics permitting reliable analysis of multiple lipid species within short analysis time. The accurate quantification of individual lipid species provided new insights into membrane structure, lipid metabolic pathways and metabolic flux [151,152]. Lipidomics of the stress response in yeast uncovered the essential function of sphingolipids in heat protection coupled to their signaling role in Hsp production [153–156]. The role of PLC and PLD in membrane-related stress signaling events in plants was identified by these modern technologies [157–160]. In mammalian systems, lipidomics revealed pronounced and highly specific alterations in lipid composition, pointing out the role of PLA_2, PLC and sphingolipid metabolism during stress-induced membrane perturbations [161,162]. The plethora of new data requires new approaches for bioinformatic. Supervised and unsupervised classification methods, such as discriminant, cluster and principal component analysis or other pattern recognition techniques [163] together with the integration of lipidomics with transcriptomics, proteomics and metabolomics [164] are introduced in order to understand the biological role of lipid networks in stress management [150].

In summary, stress-lipidomics is developing rapidly as an emerging field and by its methodological innovations it is available to detect and

monitor fine details of membrane lipid changes and the associated production of signaling metabolites.

7. Bridging the gap between plasma membranes and heat shock genes: Heat Shock Factor 1 at the crossroad

7.1. Hsp expression is regulated by HSFs

Inducible expression of Hsps is controlled by members of the HSF family. So far, four members are identified in vertebrates: HSF1 and HSF2, which exist in all vertebrates, HSF3, which is present only in avian species, and HSF4, which is only present in mammals [165]. Although both HSF1 and HSF2 are involved in the heat-inducible regulation of Hsps, most research has focused on HSF1.

Under optimal growth conditions, HSF1 shuttles as a latent monomer between the cytoplasm and the nucleus in complex with Hsp90, p23 and immunophilin. Upon elevated temperatures, this complex disintegrates; HSF1 accumulates in the nucleus, quickly undergoes several post translational modifications, trimerizes as a homo- or heterotrimer together with HSF2 [166] and binds to its recognition site (heat shock element, HSE) in Hsp-promoter region and ultimately drives Hsp-expression [167].

7.2. HSF1: a central hub of membrane-mediated stress signaling

Our understanding of how exactly the signal from heat perception at the plasma membrane is transduced toward HSF1 activation, ultimately driving the expression of Hsps, is still incomplete and fragmentary. However, over the last decades a complex interplay of multiple pathways emerged in which derivatives of plasma membrane phospholipids, sphingolipids and cholesterol all play important roles (Fig. 5). Of these, PIP2 and ceramide play a central role in the signaling transduction. For ease of interpretation, the signaling cascades originating from the plasma membrane and activated upon heat stress can be grouped according to the central second messenger lipid moieties driving the cascade. In fact, PIP2 is the central driver of both the inositol triphosphate/diacylglycerol signaling and the phosphatidylinositol trisphosphate signaling cascades. Ceramide on the other hand is at the middle of sphingosine- and cholesterol-dependent signaling pathways. Next, sphingosylphosphorylcholine (SPC)-mediated signaling constitutes an additional cascade which targets Hsp expression. Ultimately, most if not all, of these signaling cascades affect the activity of HSF1, while a lot of potential crosstalk exists between these signaling cascades. In the following paragraph, the different signaling cascades will be outlined in more detail.

Recently, ligand-independent activation of the epidermal growth factor receptor (EGFR) was shown upon lipid raft disruption [168]. It was assumed that cholesterol depletion from the plasma membrane by MBCD leads to the release of EGFR from the damaged raft into small confined areas of the membrane, where the receptor molecules are likely to be spontaneously activated owing to a very high density and/or separation from the inhibitory factors remaining in the surrounding portions of the membrane. In addition, Simons and coworkers most recently demonstrated, that the plasma membrane ganglioside GM3 alone, exhibits the potential to regulate the allosteric structural transition from inactive to a signaling EGFR dimer, by preventing the autophosphorylation of the intracellular kinase domain in response to ligand binding [169]. Based on these findings, the "membrane sensor" model hypothesizes that signaling from the transmembrane growth factor receptors to *hsp* genes can link plasma membrane events to Hsp expression induced by mild heat and/or membrane fluidizers [170]. As such, membrane rearrangement by mild heat or chemical fluidizers may activate growth factor receptor tyrosine kinases by causing their non-specific clustering [147].

Indeed, exposure to mild heat in a number of cell lines was shown to activate EGFR [171]. Activation of GFR results in activation of PLC [172] and phosphatidylinositol-3-kinase (PI3K) [1], triggering the inositol triphosphate/diacylglycerol and phosphatidylinositol triphosphate signaling cascades, respectively. In fact, active PLC hydrolyzes PIP2 into inositol triphosphate (IP3) and diacylglycerol (DAG) [173]. IP3 binds to IP3 receptors (IP3R) – intracellular Ca^{2+} channels – causing the release of Ca^{2+} from intracellular Ca^{2+} stores such as the endoplasmic reticulum and Golgi apparatus. Of note, the heat-induced generation of IP3 and subsequent Ca^{2+} influx were shown to be decreased in PLC-deficient *Arabidopsis* mutants, while the expression levels of the small Hsps Hsp18.2 and Hsp25.3 were decreased [174]. Subsequent release of Ca^{2+} into the cytoplasm has multiple effects including the activation of calmodulin kinase II (CaMKII), via the interaction with calcium–calmodulin [175], and the activation of cytosolic phospholipase A_2 ($cPLA_2$) [172]. Active CaMKII results in phosphorylation of HSF1 while $cPLA_2$ drives the formation of AA. In parallel, the initial generation of DAG by PLC from PIP2 leads to PKC activation and the additional formation of AA by the DAG lipase–MAG lipase pathway [161]. DAG lipase is activated by phosphorylation by PKC and PKA while the molecular mechanisms by which MAG lipase is activated are still elusive [176,177]. Activation of PKC contributes to cPLA2 activation [178], which – either directly or indirectly via the activation of the MAPK pathway – activates HSF1. AA formation also contributes to the activation of HSF1 [162,179].

In parallel, GFR kinases result in the activation of PI3K leading to the production of phosphatidylinositol-3,4,5-triphosphate (PIP3) from PIP2 and subsequent parallel activation of AKT and Rac1 [180]. The serine/threonine kinase AKT links growth factor signaling with Hsp response by both inhibiting the glycogen synthase kinase-3 (GSK3) and activating mTOR both of which result in subsequent HSF1 activation [181,182]. In parallel, Rac1 plays a fundamental role in a wide variety of cellular processes including Hsp response [183]. Indeed, during moderate heat shock, activation of PI3K has been observed [184] followed by activation of AKT [181] and Rac1 [183] ultimately resulting in enhanced Hsp expression levels.

Next to GFR-related signaling in HSR signaling, an essential contribution of TRPV1 channels for efficient induction of the HSR upon heat was recently demonstrated [84]. In fact, activation of TRPV1 channels results in an extra influx of Ca^{2+} ions into the cytoplasm. Interestingly, trafficking of TRPV1 channels to the plasma membrane is mediated by PKC and PI3K while the TRPV1 channels are sensitized by PLC [185]. In addition, TRPV1 channels were shown to interact with Hsp70 suggesting the contribution of Hsp70 to the trafficking of TRPV1 to the membrane and its stability in the plasma membrane [84].

Next to the phospholipid-dependent signaling cascades discussed so far, also sphingolipid- and cholesterol-dependent signaling pathways contribute to transduction of the heat shock signal. In fact, the production of ceramide during heat stress, formed by the hydrolysis of sphingomyelin by the action of acid sphingomyelinase (aSMase), has been described and is of central importance to drive the sphingosine- and cholesterol-dependent signaling cascades [161,186]. Although the molecular mechanisms by which the activity of aSMase is regulated are not completely understood, the involvement of oxidative stress and PKC-mediated phosphorylation of aSMase have been suggested [187–189]. Next, ceramide is deacylated by ceramidase (CDase) to form sphingosine which can be subsequently phosphorylated by sphingosine kinase 1 (SK1) resulting in the formation of spingosine-1-phosphate (S1P) [190]. The activity of CDase is regulated by Ca^{2+} while the activity of SK1 is regulated by growth factor receptors via PLD signaling [190,191]. Ultimately, although a direct effect on HSF1 has not been analyzed yet, S1P activates Hsp expression via p38 MAPK and PI3K activation [192,193].

In parallel, ceramide can be glycosylated to form glucosylceramide (GlcCer) by the enzymatic activity of glucosylceramide synthase (GCS). Subsequently, the glucose moiety of GlcCer is transferred to cholesterol by the enzymatic activity of glucosyltransferase (SGT) [194]. This cascade ultimately drives the formation of cholesteryl glucoside which results in HSF1 activation [195].

Interestingly, next to driving sphingosine- and cholesterol-dependent signaling cascades, increased ceramide levels might compete with cholesterol for raft localization, ultimately forming ceramide-enriched membrane microdomains [196]. It is assumed that by altering the lipid composition of the raft, the signaling characteristics of these rafts are modulated by qualitative and/or quantitative changes of raft-residing proteins enabling the cell to better cope with a stressful environment [147].

Finally, hydrolysis of sphingomyelin by the enzymatic activity of sphingomyelin deacylase results in the formation of sphingosylphosphorylcholine (SPC) which, although a direct effect on HSF1 was not studied, ultimately drives Hsp27 expression via p38 MAPK signaling [197].

7.3. Plasma membrane modifications can modulate HSF1 activity in the absence of stress

Some of our current knowledge evidencing the central role of the plasma membrane in stress sensing comes in fact from several experiments in which lipid derivatives were shown to activate HSF1 and/or elicit a HSR in the absence of stress. Indeed, exogenous administration of cholesteryl glucoside to human fetal lung fibroblast (TIG3) cells was shown to activate HSF1 in the absence of stress [198]. In addition, exogenous administration of reactive aldehydes such as 4-hydroxynonenal (HNE) and crotonaldehyde (CRA), both plasma membrane lipid peroxidation end products, was shown to activate HSF1. In fact, HNE is the major α, β-unsaturated aldehyde derived from peroxidation of omega-6 polyunsaturated fatty acids, while CRA is an α, β-unsaturated aldehyde which is, next to being an important toxic compound of cigarette smoke and automobile exhaust, endogenously derived from peroxidation of omega-3 polyunsaturated fatty acids. Both in vitro as well as upon exogenous administration to RKO colon carcinoma cells, HNE resulted in HSF1 activation [199,200]. At the molecular level, HNE was shown to modify Hsp70, Hsp90 and the HSF1 transcription repressor Daxx, thereby disrupting the inhibitory interaction between these proteins and HSF1 [201,202], ultimately enabling HSF1 to bind its DNA recognition site and drive Hsp expression. Also, exogenous addition of CRA to human umbilical vein endothelial (HUVEC) cells was shown to cause nuclear accumulation of HSF1 leading to Hsp70 upregulation while, at the molecular level, the involvement of the c-Jun kinase pathway, the availability of Ca^{2+} ions, and the presence of reactive oxygen species were demonstrated [203]. Next to exogenous administration of lipid derivatives to cells, mechanical deformations of the plasma membrane, caused by mechanical stress, were equally shown to result in HSF1 activation. Indeed, in both synovial fibroblast-like cells (SFC) and in atherosclerotic lesions from an animal model of arthritis, active HSF1 was observed [204,205]. Moreover, mechanical stretching of smooth muscle cells resulted in HSF1 translocation from the cytoplasm to the nucleus and hyperphosphorylation followed by increased Hsp70 expression [204].

While being at the first stage of stress sensing, the plasma membrane might actually represent a tool allowing us to modulate HSF1 activity ultimately influencing the outcome of HSR. Considering that a deregulation of the HSR is central to several high prevalence disease states such as cancer and neurodegenerative diseases, the ability to influence the HSR by modulating HSF1 activity is indeed of high clinical relevance. In the next paragraph, we will outline how membrane fluidization and membrane intercalating hydroximic acid derivatives

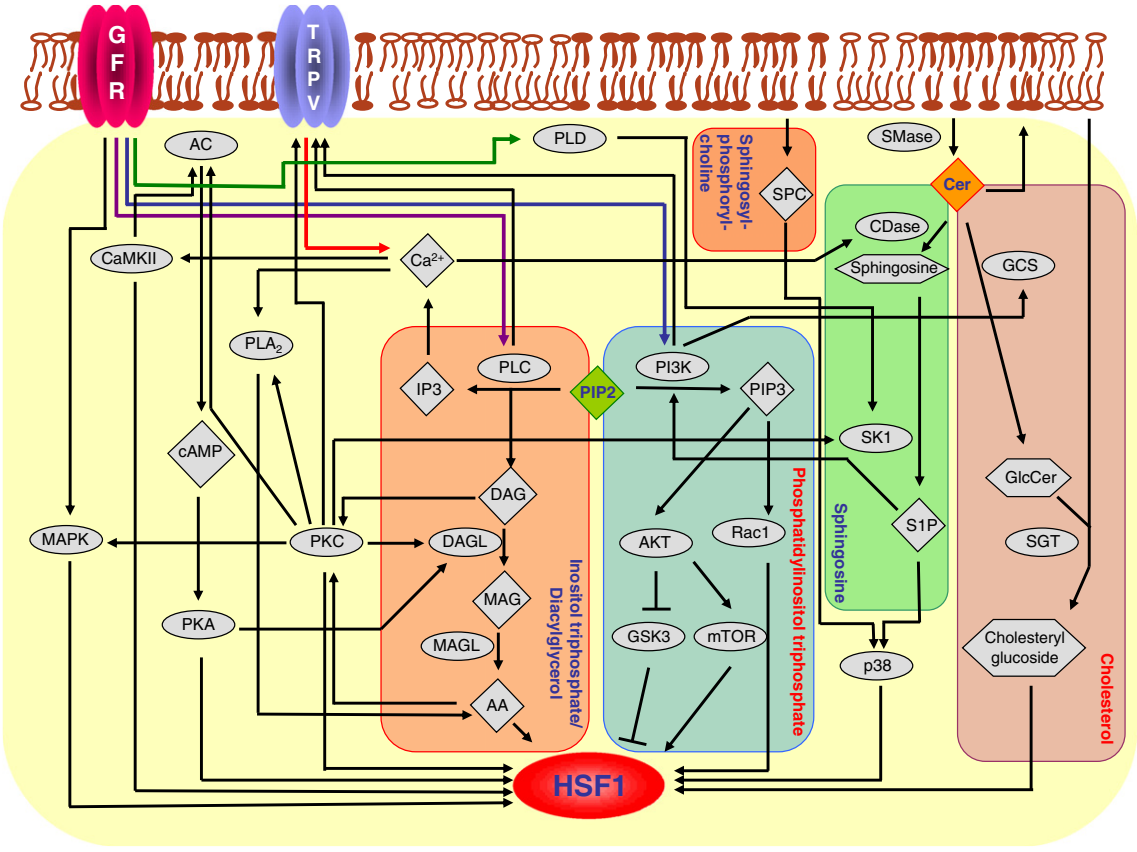

Fig. 5. HSF1 is targeted by multiple membrane-originating stress-induced signaling cascades. The signaling cascades originating from the plasma membrane and activated upon heat stress can be grouped according to the central second messenger lipid moieties driving the cascade. PIP2 is the central driver of both the inositol trisphosphate/diacylglycerol signaling and the phosphatidylinositol triphosphate signaling cascades. Ceramide is at the middle of sphingosine and cholesterol-dependent signaling pathways. Sphingosylphosphorylcholine (SPC)-mediated signaling constitutes an additional cascade which targets Hsp expression. See text for details.

modulate HSF1 activity and, as such, might represent future strategies for targeted HSR modulation.

7.3.1. Plasma membrane fluidization by benzyl alcohol activates HSF1

By intercalating between membrane lipids, BA causes a disordering effect due to weakening of the van der Waals interactions between the lipid acyl chains. As such, it was demonstrated in human erythromyeloblastoid leukemia K562 cells that BA is able to induce similar changes in membrane fluidity comparable to that induced by mild heat stress exposure in a dose-dependent manner [113]. Consequently, BA was able to induce a heat shock response at the normal growth temperatures in the absence of general proteotoxicity, while causing a downshift in the temperature threshold of the heat shock response. Interestingly, BA was able to provoke a distinct HSR at growth temperature comparable to heat stress [99]. At the cellular level, a Ca^{2+} influx similar to that seen during heat shock was observed in parallel with mitochondrial membrane depolarization [113]. Subsequently, it was shown in mouse embryonic fibroblast cells (MEF) that Hsp-induction by BA was dependent on HSF1 activation [99]. Interestingly, in mouse melanoma B16 cells, it was found that, while a general increase in plasma membrane fluidity was observed, BA specifically caused structural changes in the microdomain (raft) structure [99]. Based on these findings it was suggested that rather than plasma membrane fluidization per se, a specific reorganization of cholesterol-rich raft structures might be responsible for the generation and transmission of stress signals ultimately resulting in Hsp gene activation.

7.3.2. Plasma membrane lipid raft intercalating compounds modulate HSF1

Hydroximic acid derivatives, including BGP-15 and BM, are well established Hsp co-inducers [8]. For example, the multi target drug BGP-15 has proven clinical effects in animal models of muscular dystrophy, atrial fibrillation, and type 2 diabetes [8]. Although the precise molecular mode of action of BGP-15 is not yet completely clear, it has recently been shown that BGP-15 is able to increase the stability of cholesterol/sphingomyelin complexes in in vitro monolayer experiments [127]. Furthermore, upon labeling of lipid rafts with mGFP-GPI, it was shown in B16 cells that BGP-15 was capable to preserve the integrity of these rafts when challenged by thermal stress [127]. Interestingly, in parallel to the previous observation with BA [99], this study revealed that BGP-15 also affected the size and the distribution of lipid rafts in heat shock response-deficient B16 cells while restoring the capacity of these cells to upregulate Hsps upon heat exposure [127]. Based on these observations, it was suggested that raft reorganization is coupled with the triggering of raft-associated stress sensing and signaling pathways. Considering the previous observations that BM – a BGP-15 predecessor – could prolong the activation of HSF1 [206] combined with a report suggesting that acetylation of HSF1 attenuated the HSR [207], the effect of BGP-15 on HSF1 acetylation was analyzed. As such, it was demonstrated that pre-treatment of human embryonic kidney (HEK293) cells with BGP-15 resulted in reduced HSF1-acetylation suggesting prolonged activation of HSF1 [127]. Taken together, by intercalating into the plasma membrane and causing lipid raft remodeling, it is suggested that BGP-15 might be able to co-induce the HSR by reducing the total amount of HSF1 acetylation.

8. Genetic modification of a pathogen's membrane physical state alters the heat shock response and causes loss of virulence of *Salmonella* Typhimurium LT2 and *Histoplasma capsulatum*

In microorganisms, plants and other poikilotherms (organisms whose body temperature varies with the environmental temperature) gradual changes in growth temperature modify the cell's membrane physical state (MPS) [208] resetting the threshold temperature at which Hsps are synthesized under stress conditions [5,209]. It has been shown that under physiological temperatures, MPS, regulated by the environmental temperature, is controlled by lipid unsaturation, protein–lipid ratio, composition of lipid molecular species, etc., that determine the temperature at which heat shock genes are transcribed [5,210]. One of the factors in regulating the MPS is represented by fatty acid desaturases (Δ^5, Δ^9, Δ^{12}, etc.), enzymes which introduce a double bond in a specific position in long-chain fatty acids [211]. Further, the unsaturation of fatty acids in glycerolipids is essential for the proper functioning of biological membranes.

We have studied the role of the MPS during the Mϕ infection in the important human and animal pathogens *S. enterica* serovar Typhimurium and in the human fungal pathogen *H. capsulatum* whose mechanisms of virulence are well characterized. We have shown that in *S. typhimurium* the alteration of the MPS induced by expression of heterologous *Synechocystis* Δ^{12}-desaturase or its trans-membrane spanning regions alters the pattern of Hsp accumulation, which is of significant biological importance for the organism's pathogenicity.

8.1. Effects of expression of heterologous Synechocystis Δ^{12}-desaturase on membrane physical state of S. typhimurium

We had demonstrated that the primary sensor of temperature variations and, of other forms of stresses is localized in the plasma membrane [210,212]. More recently, we and others have shown that a heat shock or an exposure to other stressors determines a physical reorganization of lipid and protein membrane components [4,147,150,213] that is followed by a specific gene response designed to, among other things, compensate variations in the MPS [3,214]. Fluidity and microdomain organization of membrane are strictly involved in the perception and transduction of stressors (e.g. heat) into secondary signals that induce transcription of specific heat shock genes [215,216]. Under stress conditions, a reversible association of specific Hsps with membranes may re-establish their fluidity and bilayer stability and, thereby, restore membrane functionality.

We have studied the role of the membrane during Mϕ infection in *S. typhimurium* and showed that an abrupt change of MPS alters the pattern of Hsp accumulation, which eventually has profound biological significance for the organism's pathogenicity [217]. *S. typhimurium* genome does not contain any Δ^9-fatty acid desaturase (*S. enterica* subsp. *enterica* serovar Typhimurium str. LT2: http://www.ncbi.nlm.nih.gov/genome/152?project_id=57799), hence, it does not produce oleic acid (18:1) which is the natural substrate for Δ^{12}-fatty acid desaturase. Expression of *Synechocystis* Δ^{12}-desaturase in *S. typhimurium* caused a significant imbalance in MPS due to the higher protein/lipid ratio and membrane leakage. The protein which is a membrane-bound enzyme [218] has no enzymatic activity in *Salmonella*, thus, its perturbing effect on the MPS is due to its insertion in the membrane rather than to a change in phospholipid composition.

The fatty acid chain length was similar in control strain and transformed *S. typhimurium*, though lipid unsaturation decreased of ca. 20% in *S. typhimurium* expressing either the entire Δ^{12}-desaturase coding sequence or the two trans-membrane regions of the protein. The insertion of the peptides or the full protein caused a change in MPS while cells compensated for the increased fluidity by reducing the unsaturation of their membrane lipids. The most significant change was a decrease of 16:1 and the increase of 18:0 fatty acids.

Further, perturbation of membrane functionality and destabilization caused by the insertion of Δ^{12}-desaturase were determined by measuring the uptake of 1-N-phenylnaphthylamine (NPN) fluorophore, which correlates with membrane permeability. The perturbation was significant in the normal temperature range of 25 to 40 °C while it was not detected at higher temperatures. Such results were confirmed by differential scanning calorimetry on isolated membrane. Furthermore, insertion of Δ^{12}-desaturase in membrane lowered the transition temperature of certain lipid domains in the outer membranes of *Salmonella*. Therefore, *S. typhimurium* expressing Δ^{12}-desaturase gene had greater permeability in the outer membrane, under stress and non-stressing conditions. Recently, Yamamoto and Ando have shown that endothelial

cells directly respond to shear stress by a rapid decrease of their lipid phase order and postulated that these changes could be linked to shear stress sensing and to response mechanisms [219]. Possibly, the altered membrane organization induced a compensatory mechanism that may comprise alteration of the ratio of gel-fluid lipid domains [220].

Furthermore, when *S. typhimurium* was incubated for 30 min with different concentrations (between 5 and 80 mM) of BA and then plated on LB agar, no toxic effect was detected up to 50 mM BA on cell viability [113,212]. We and others had shown previously that BA and heptanol, both membrane fluidizers, do not cause protein denaturation at concentrations that induced the HSR [221] at non-inducing heat shock temperatures, implying that the transcription of heat shock genes was due to their interaction with the membrane [4].

Insertion of the whole Δ^{12}-desaturase or its trans-membrane peptides perturbed significantly the MPS of *Salmonella* membrane and reset the optimal temperature of expression of heat shock genes. Fig. 6 shows that, while in normal cells a heat shock between 38 and 47 °C caused a progressive increase of the small heat shock proteins dnaK and ibpB transcription, transformed cells had a reverse pattern which was reduced at high temperatures. In addition, ibpB was overexpressed in transformed *Salmonella* at physiological temperature, suggesting that insertion of Δ^{12}-desaturase protein in the membrane fraction induced a complex compensatory mechanism that included alteration of the phase transition temperature of certain lipid domains [5,147]. These results were further corroborated by MALDI [222] and MS-Fit database analyses [223] that showed association of IbpA and IbpB to the outer membrane fraction. Thus, the behavior of *Salmonella* in response to the expression of the entire Δ^{12}-desaturase gene is, at least in part, due to the insertion of the protein in its membrane supporting our model that comprises a cross talk between changes in MPS and transcriptional regulation of genes involved in lipid metabolism and in HSR [3,224].

However, the effect of perturbation by Δ^{12}-desaturase (or its trans-membrane domains) on the MPS and the HSR can be obtained also by using other membrane proteins unrelated to fatty acid metabolism or desaturation, as we have shown by expressing α-crystallin in eukaryotic cells. Further, we have shown recently by RNAseq analysis that the expression of one of the two trans-membrane regions of Δ^{12}-desaturase gene alters the pattern of expression not only of heat shock genes but also of genes that are involved in the adaptation to conditions present in Mϕ and in virulence (Porta et al., unpublished data). These changes are likely due to the insertion of the trans-membrane spanning regions of two-component histidine kinase sensors (e.g. PhoP/PhoQ [225]; PmrA–PmrB; [226]).

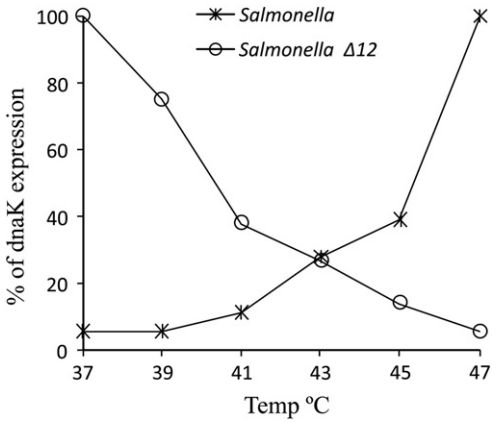

Fig. 6. Pattern of expression of dnaK in wild type (*Salmonella*) and genetically modified *S.* Typhimurium (*Salmonella* Δ12) grown at 37 °C. dnaK was poorly expressed at 37 °C in wild type cells but was highly transcribed in the genetically modified strain. When cells were heat shocked between 39 and 47 °C, dnaK expression increased up to 47 °C while it progressively decreased up to 45 °C and was poorly transcribed at 47 °C.

Thus, the imbalance in the membrane lipid/protein ratio, due to overexpression either of Δ^{12}-desaturase or individually expressed trans-membrane regions of this protein, caused major changes in MPS and a significant reduction of the HSR, implying that in *S. typhimurium* the perception of temperature is strictly controlled by membrane order and by a specific membrane lipid/protein ratio. Further, the capacity to control the level of Hsps allows the possibility to produce pathogens with a reduced capacity to adapt to the host environment at the onset of infection [227].

8.2. Attenuation of virulence, membrane fluid state and heat shock response of *H. capsulatum*

So far, a methodology to obtain live attenuated strains has not been obtained for fungal pathogens. Live attenuated strains of human protozoa such as *Toxoplasma*, *Leishmania* or of other parasites to develop vaccines have been produced. However, so far none of these candidate vaccines has been considered suitable for clinical use [228,229]. Recently, an alternate strategy to vaccines based on purified antigens has been used exploiting the notion that low-density infections induce antibody-independent immunity to different strains of *Plasmodium* [230]. These authors treated parasitized red blood cells from the rodent parasite *Plasmodium chabaudi* with seco-cyclopropyl pyrrolo indole analogs, drugs that irreversibly alkylate parasite DNA, blocking its replication. Immunity was mediated by $CD4^+$ T cells and was dependent on the integrity of red blood cell membrane. These experiments demonstrated that vaccination with chemically attenuated parasites induces protective immunity.

However, a method to obtain live attenuated fungal pathogens has not been found. The production of genetically modified pathogens that lack virulence or essential genes is considered the main alternative for developing an effective protective vaccine. Present genetic procedures are based on the use of mutations or deletions of specific (virulence) genes that may cause attenuation and are species-specific. Thus, a given method of attenuation may be effective in a specific organism but not in other pathogens.

We had shown previously that modulation of the MPS of the human fungal pathogen *H. capsulatum* by treatment with BA [212] or by alteration of the saturated/unsaturated (SFA/UFA) ratio caused a reduced HSR. These results encouraged us to investigate the effect of reduced levels of Hsps on the virulence of this pathogen at the onset of infection in Mϕ and in a mouse model of infection by overexpressing a homologous Δ^9-desaturase gene in the highly virulent G217B strain of this fungal pathogen.

8.3. *H. capsulatum* Hsp70 gene expression, MΦ and mouse infection

We constructed a G217B mutant strain carrying an extra copy of its own Δ^9-desaturase gene under the transcriptional control of the up-regulated Downs Δ^9-desaturase promoter [231]. The parental strain expresses Hsp70 between 34 and 42 °C with a maximum at 37 °C, the genetically modified strain did not express this gene at 37 °C, but at the higher temperature of 42 °C. This result was similar to that of wild type strain treated with oleic acid (an UFA) or BA in which Hsp82 transcription was hampered. The mutant strain, as well as the strain treated with BA or with an UFA, had a more fluid membrane. *H. capsulatum* G217B and the genetically altered strain were added to a Mϕ monolayer at 37 °C to test their capacity to survive within these cells that constitute the natural environment that allow growth of 'yeast' phase cells (the pathogenic form of the dimorphic *H. capsulatum*) in a susceptible mammalian host. Contrary to control cells, the altered strain after internalization did not grow inside Mϕ and no fungal cells were recovered after 8 h of infection.

Increasing concentrations of yeast phase cells of the virulent G217B or of the genetically modified *H. capsulatum* strain (10^6, 10^7, and 5×10^7) were injected intravenously into CD-1 mice. All mice injected

with the modified strain or with control cells at a concentration of 10^6–10^7 survived up to 40 days. Mice infected with the higher dose of yeast cells of the virulent G217B strain (5×10^7) died within 6 days, while 7 out of 10 mice infected with the same inoculum of the modified strain survived up to 45 days after infection (Fig. 7). Forty days after the initial infection with the modified strain, the surviving mice were re-infected with a lethal dose (5×10^7 yeast cells) of the virulent G217B strain. Sixty per cent of the initial group of the pre-treated mice (60 days from the initial infection) 20 days after infection still survived, while all mice injected, as control in parallel with the virulent strain died within 6 days after the inoculum (Fig. 7).

In conclusion, Hsps have been implicated in the stimulation and generation of both innate and adaptive immunity in a wide variety of microorganisms, eliciting humoral responses during natural infection with various pathogens. Anti-Hsp70 antibodies have been detected in patients with chronic parasitosis and Hsp70 of *Plasmodium falciparum*, *Schistosoma mansoni*, and *H. capsulatum* have also been shown to induce a cell-mediated immune response [232]. A putative role for Hsp90 in immunity has also been proposed [233]. However, vaccination with recombinant proteins does not protect mice, thus, though Hsps have antigenic properties, they do not mediate protection. Our approach is based on the alteration of the HSR to reduce rather than stimulate the synthesis of Hsps, to generate a strain defective in the process of stress adaptation. Intracellular pathogens at the onset of infection induce the transcriptional activation of stress genes along with other species-specific genes, broadly defined as virulence genes. The latter class of gene products is involved in the mechanisms of invasion/adaptation, and is responsible for the capacity of the pathogen to invade, replicate, and induce disease in the host [234]. Thus, expression of stress genes and those involved in virulence is temporarily associated and transcriptionally coordinated [235].

We have shown that it is possible to obtain with a single genetic modification a new procedure to produce an attenuated strain from the highly virulent fungus *H. capsulatum* by repressing the HSR. We have modified *H. capsulatum* with a gene involved in the regulation of the MPS, causing impairment of the pathogen's stress response that causes a parallel failure of the pathogen to survive within MΦ (as shown in *Salmonella* in a previous section) and to cause disease in a mouse model of infection. Further, the genetically-modified and attenuated *H. capsulatum* strain induced protection in mice after subsequent challenge with a highly virulent strain.

More recently, we have used the *Salmonella* strain expressing a trans-membrane region of *Synechocystis* Δ^{12}-desaturase gene and tested its efficacy in a murine model of infection (Porta et al., unpublished). We obtained similar positive results. Further, we have monitored the immune response during infection and analyzed by RNAseq the profile of mRNA transcription of the modified *Salmonella* strain. We could show that the strain induced a full immune protection and an altered HSR as well as a strong impairment of transcription of virulent genes.

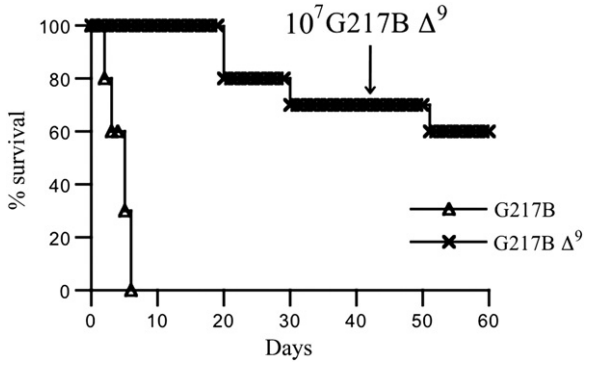

Fig. 7. Mouse infection with *H. capsulatum*. Mice infected with 5×10^7 G217B yeast cells died within 6 days, while 70% of mice infected with the same inoculum of genetically modified strain (G217B Δ^9) survived up to 40 days after infection. Then surviving mice were challenged with a lethal dose of the virulent strain G217B (5×10^7). After 20 days (60 days from the initial infection with attenuated genetically modified strain) 60% of mice still survived.

Since this procedure is effective both in prokaryotes as well as eukaryotic pathogens, it may be possible that attenuation of virulence obtained by modification of MPS and HSR might be applied to other very important intracellular pathogens, such as mycobacteria, staphylococci, streptococci, *Pseudomonas* and parasites.

9. From bench to bedside: therapeutic applications of heat shock protein-modifying agents

Since Ritossa's first observation fifty years ago that heat shock induced swelling on a *Drosophila* chromosome which led to the discovery of heat shock proteins, approximately 50,000 publications have been published on these stress-protecting proteins [236]. While basic science research offers a major understanding of how the proteins aid survival and robust living, therapeutic application of Hsp induction to human disease states remains a promise and not a reality. We believe that we are at the dawn of a renaissance in drug discovery. A host of previously untreatable or inadequately treated diseases are likely to be prevented or at least modified beneficially to lessen the impact on health.

As cyto-protective agents, Hsps enable cells and thus organ systems to resist as well as recover from stress. Hsps preserve cellular organelles, delay or prevent apoptosis, reduce oxidative and free radical damage, limit inflammatory response, aid in immune tolerance or augment immune activation, remove irreversible damaged cellular proteins, and enhance the gut-body barrier.

Studies in the late 1980s revealed that a brief febrile range heat shock that raised cellular Hsps could condition an animal to better tolerate an insult. In particular, Barbe, Tytell and coworkers observed that 15 min of hyperthermia at 41 °C induced a marked decrease in photoreceptor damage after exposure to bright light as compared to normothermic animals [237]. New studies demonstrate that hyperthermia preconditioning can improve survival from sepsis [238], stroke [239], myocardial infarction [240], hepatic ischemia [241], surgical wound healing [242], surgical tendon repair [243], and organ transplant survival [244]. Hyperthemia is also therapeutic in osteoarthritis [245], type 2 diabetes (glycemic control, neuropathy and nephropathy) [246,247], and in some cancer therapies [248]. While whole body hyperthermia or localized heat application holds a real therapeutic option in certain diseases, its practical application is limited by time constraints and availability. However, the heat studies support the notion that major pathologies are amenable to heat therapy and support the concept that agents mimicking the biologic effect of heat might also be effective therapies.

Membrane remodeling agents may act like heat by alarming the cell that its membrane bilayer is “melting” and thereby initiate the heat shock stress response. Indeed, we earlier reported that the thermal shift of membrane fluidity induced by heat is duplicated by membrane fluidizers such as BA and heptanol [150]. Diverse Hsp inducers and co-inducing agents have been studied in animal disease models. While not all of these agents alter membrane fluidity, their efficacy in treating pathology via Hsp induction predicts the potential for unrelated Hsp inducers to be similarly capable of therapeutic benefit.

An array of diseases is associated with inadequate or decreased Hsp response. Deficient Hsp conditions include aging itself [249], certain acute catastrophic diseases (sepsis [250], acute respiratory distress syndrome [251], extensive body burns [252], pancreatitis [253]), chronic illnesses (diabetes (both types 1 and 2) [254,255], colitis [256], psoriasis [257], neuro-degenerative disease [258,259], renal failure [260], cigarette smoking [261], and chronic obstructive lung disease [262]).

Additionally, genetic deficiencies of Hsps, chaperonopathies, have been described: Bardet–Biedl syndrome (BBS), neuropathies associated with mutations in small Hsps, Williams syndrome (WS), dilated cardiomyopathies (DCs), and ataxia of Charlevoix–Saguenay (ARSACS) [263].

Beyond the Hsp deficiency, these diseases are characterized by excessive accumulation or misfolding of proteins that can lead to malfunction and cellular demise. Many of these diseases are associated with aging and neurodegeneration, including Alzheimer's, Parkinson's, amyotrophic lateral sclerosis (ALS), and certain muscular dystrophies. Atrophic states like macular degeneration and retinitis pigmentosa appear to respond to anti-trophic Hsp agents [264].

Acute injury events benefit from augmented stress response: post stroke, myocardial infarction, trauma, auto accidents or surgery, renal dialysis, retinal detachment, sunburn and heat stroke. As organs lose function, due to disease or life events, a heightened stress response could beneficially limit further organ damage or even restore it (heart, kidney, liver, skeletal muscle, retina). Finally, Hsp-inducing agents aid tolerance and survival of toxins (acetaminophen (APAP) over dose [265], chemotherapy [266], excessive alcohol consumption [267]).

Autoimmune diseases such as type 1 diabetes and adjuvant-induced arthritis can be modified by Hsp exposure or induction. Hsps increase T lymphocyte regulatory cells that ultimately reduce intolerance to self-antigens — like to the pancreas beta cell or to the joint synovia [268,269].

Cancer therapies can benefit from pharmacologic manipulation of Hsps in several ways. First, toxicity due to oncologic therapies can be limited by Hsp induction. Second, some of the agents like the hydroximic acid derivative BGP-15 and alpha lipoic acid (LPA) reduce tumor volume [270,271]. Finally, Hsp expression in cancer is often elevated in tumors and is associated with enhanced metastatic potential. Agents that reduce cancer Hsp levels can effectively be used as oncolytics. Provocatively, induction of unique viral infection can reduce tumor Hsp expression and result in oncolysis while maintaining organism well being [272]. With cardiovascular disease as the major contributor to mortality in Western Society, it is important to address how endothelial function can be maintained as endothelial dysfunction and loss of nitric oxide production is viewed as the hallmark of vascular disease. Relevantly, heat therapies as well as the TRPV activator capsaicin increase nitric oxide production by the endothelium and can, indeed, reduce ischemic vascular events [273,274]. Recent studies indicate that the heat sensor TRPV activation releases intracellular calcium that is then key in endothelial nitric oxide release. TRPV activation blocks attenuate the increased vascular tone of norepinephrine on the thoracic aorta [275].

To date, the major Hsp inducers are the hydroximic acids, geranylgeranylacetone, LPA, xenohormones, heme oxygenase inducers and chemical chaperones. Geranylgeranylacetone, a membrane-fluidizing compound, was shown to restore the membrane fluidity that was compromised by nonsteroidal anti-inflammatory drugs [276]. Table 3 demonstrates the diverse diseases with therapeutic effect in animal models of disease or in actual diseases with these agents. Often, these agents are effective with oral ingestion and have limited toxicity or side effects.

Nutritional substances from plants have long been used by all cultures to treat disease. Animals can take advantage of stress-generated bioactive products of plants, termed xenohormesis. The xenohormones can, when ingested, improve health and fitness by activating the animal's cellular stress response. In particular, the plant substances that raise Hsps and are associated with extension of life span are resveratrol, curcumin, and salicylate. Plant compounds that raise Hsps but currently have no proven longevity effects are rosmarinic acid, ferulic acid, jasmonic acid, and carvacrol [277]. A combination of Hsp-inducing herbs has been formulated in Scandinavia, Adapt 232 (iherb.com), which has been studied in multiple large, placebo-controlled human studies. As such, it is known that Adapt 232 is associated with improvement in well-being, exercise endurance, and memory in patients with chronic fatigue syndrome [278]. Similarly, an ethanol extract from prickly pear cactus (Prepair, TEX-OE) raises Hsps and improves exercise endurance, reduces alcohol hangover symptoms, aids in sperm preservation of fish for breeding, and improves survival in animal transport [279,280].

Taken together, the impact of disease on people's lives is incalculable. Imagine a family devastated by a disease like autosomal retinitis pigmentosa. Given that their blindness does not appear until adolescence or later—an intervention like an Hsp inducer could modify the disease process and prevent vision loss and secondary sequela like depression and suicide. To be able to treat diverse diseases amenable to Hsp modifying agents could lessen human tragedy and ultimately lead to higher quality, more enjoyable, longer lasting lives.

10. Concluding remarks

Over the last decade, multiple studies have suggested that cells have the ability to sense and respond to stress signals through the activation of membrane-associated signal transduction pathways. As the integrity of membrane nanoplatforms strongly depends on the thermally-controlled lipid-phase behavior, it is assumed that even mild changes in temperature might result in a redistribution and modified activity of potential stress-sensing/signaling proteins within these subdomains. Here, based on single molecule tracking, we provide new evidence indicating that even a fever-type temperature stress results in distinct structural changes to the plasma membrane. Interestingly, the fever-type temperature-induced membrane changes also alter the interacting behavior of membrane-residing proteins in a cholesterol-dependent manner, suggesting modulations of raft residing proteins, which potentially alter the signaling behavior of the nanoplatforms. In fact, the thermally gated TRP channels represent one of the potential membrane-residing heat sensors whose activity is lipid-modulated in several ways, including directly affecting the channel properties, acting as allosteric modulators altering the sensitivity to other agonists, or by indirect effects in which changes in the mechanical properties of the membrane lead to

Table 3
Therapeutic Hsp inducers and disease states responsive to therapy.

Hsp inducer	Disease State
Hydroximic acids	Insulin resistance and diabetes [281,282] and its complications (neuropathy [283,284], retinopathy [285], wound healing [286]) ischemia reperfusion [287], APAP toxicity [265], chemotherapeutic neuropathy [288], intracranial hemorrhage [289], atrial fibrillation [290], hepatoma [271], sun burn [291], vascular hypertension damage [292], myocardial infarction [293], amyotrophic lateral sclerosis [294], brain hypoxia [295], pancreatitis [296], ethanol intoxication [297], muscular dystrophy [298]
Geranylgeranylacetone	Atrial fibrillation [299], bleomycin-induced pulmonary fibrosis [300], drug induced gastritis [301], cardiomyopathy [302], myocardial infarction [303], post-surgical survival [304], autoimmune uveoretinitis [305], intra-cerebral hemorrhage [306], cerebral infarction [307], colitis [308], APAP toxicity [309], acoustic injury [310], endotoxin shock [311], glaucoma-induced retinal damage [312], retinal detachment [313], influenza infection [314], heat stroke [315], and insulin resistance [316].
Alpha-lipoic acid	Insulin resistance [317], diabetic retinopathy [318], neuropathy [319], cardiomyopathy [320], periodontitis [321], postmenopausal bone loss [322], fatty liver [323], renal vascular hypertension [324], pesticide poisoning [325], cancer [270], cerebral ischemia [326], post-surgical fibrosis [327], serum lipids [328], drug toxicity [329]
Hemeoxygenase inducers	Insulin resistance [330], type 1 diabetes [331], diabetic nephropathy [332], ischemia reperfusion in diabetic heart [333], diabetic cardiac dysfunction [334,335], fatty liver [334], bone mass [336], drug renal toxicity [337], renal vascular hypertension [338]
Chemical chaperones	Insulin resistance [339], Fabry disease [340], Gaucher disease [341,342], cardiac fibrosis [343], pancreatitis [344], Batten disease [345,346], cancer [347], protein folding diseases [348], macular degeneration [349]

alterations of the channel gating. Surprisingly, the effect of stress steroids, in altering membrane fluidity and stimulating membrane microdomain restructuring could influence stress response on the base of the membrane thermosensor concept. This establishes a nice link between the organismal stress response and the cellular stress response originating at the level of membrane sensing. Computational techniques such as molecular dynamics simulations allow us to gain insight in the lipid environment without the use of bilayer-perturbing probes. Exploiting MD, here we report that established compounds having a pronounced effect on the expression of Hsps (BA, PhA, and BM) are capable of altering lipid membranes, in a manner similar to thermal stress.

Once the plasma membrane-originating heat-induced signal is generated, it is transduced toward HSF1, ultimately driving the expression of Hsps via a complex interplay of multiple pathways. Prominent, membrane associated signaling cascades playing key role in the regulation of HSF1 activity and thereby the expression of Hsps are summarized.

Considering that multiple diseases are associated with inadequate or decreased Hsp response, plasma membrane integrity modulating tools that are able to influence the HSR were documented. Hsp-modulating strategies centering on (plasma) membrane modulations are potentially of high therapeutic value. The mode of action of membrane-intercalating Hsp-modulators fully supports the "membrane thermosensor" concept which has great potential for use in 'membrane-lipid therapy'. Beyond classical pharmacological interventions, here we also present evidence that by genetically modifying the membrane physical state of *Salmonella* Typhimurium LT2 and *H. capsulatum*, the HSR can be altered concomitantly resulting in a loss of virulence of these pathogens.

By the application of high-content ultrasensitive methods, including image based cell profiling and/or lipidomics, we can identify specific changes in membrane domain structure leading to selective refinement of the expression of Hsps. This approach can open novel avenues in the therapeutic interventions of most various diseases.

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