CMLS, Cell. Mol. Life Sci. 62 (2005) 10–23
1420-682X/05/010010-14
DOI 10.1007/s00018-004-4208-7

CMLS Cellular and Molecular Life Sciences

Review

Cellular responses to mild heat stress

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Received 14 May 2004; received after revision 2 August 2004; accepted 16 August 2004

Abstract. Since its discovery in 1962 by Ritossa, the heat shock response has been extensively studied by a number of investigators to understand the molecular mechanism underlying the cellular response to heat stress. The most well characterized heat shock response is induction of the heat shock proteins that function as molecular chaperones and exert cell cycle regulatory and anti-apoptotic activities. While most investigators have focused their studies on the toxic effects of heat stress in organisms such as severe heat stress-induced cell cycle arrest and apoptosis, the cellular response to fever-ranged mild heat stress has been rather underestimated. However, the cellular response to mild heat stress is likely to be more important in a physiological sense than that to severe heat stress because the body temperature of homeothermic animals increases by only 1–2 °C during febrile diseases. Here we provide information that mild heat stress does have some beneficial role in organisms via positively regulating cell proliferation and differentiation, and immune response in mammalian cells.

Key words. Heat stress; heat sensing; cell cycle; growth; differentiation; thermotolerance; signal transduction.

Introduction

Organisms have undergone natural selection for how to deal with the insults of thermal fluctuations in the ambient environment to obtain well-developed defense and adaptation machineries. When cells encounter heat stress, they provoke active responses such as raising signal pathways and reprogramming gene expression to retune their internal milieu. The most well characterized heat shock response is induction of a highly conserved set of polypeptides termed the heat shock proteins (HSPs) [1, 2]. The HSPs are also increased by other unrelated stresses such as oxidative and osmotic stresses and are detected even in the absence of stress [1–4]. HSPs such as HSP70 and HSP90 function as molecular chaperones that facilitate protein folding and assembly, and membrane translocation [1, 2, 5–7]. The HSPs are also implicated in cell cycle regulation [8], in resistance to stress-induced programmed cell death or necrotic cell death, and in antioxidative defense [9–17]. Expression of HSPs by heat stress is mediated by activation of the heat shock factor 1 (HSF1) [1, 2, 18–21]. Under normal conditions, HSF1 forms heterocomplexes with regulatory proteins such as HSP70 and HSP90 in the cytosol, which interfere with HSF1 transactivation [22–32]. It is generally accepted that accumulation of non-native proteins caused by heat stress is a proximal signal for HSF1 activation [1, 2, 33–37]. As a result of competition with non-native proteins for chaperones that prefer non-native proteins to HSF1, HSF1 is relieved and activated through a multistep process that involves conversion from the inactive monomer to the homotrimer, translocation into the nucleus, binding to heat shock element (HSEs), a consensus sequence located upstream of heat shock genes and target gene activation [19–21, 38–40]. HSF1 is a redox-sensitive transcriptional factor and can be directly activated by

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oxidative stresses and heat stress in vitro and in vivo, which can be reversibly inactivated by reducing agents [41–45]. Recently, it was also demonstrated that HSE is not the only HSF1 binding site in the promoters of the heat shock genes, and transcription factors other than HSF1 are also implicated in the induction of transcript that accumulates after heat stress [46].

Although information about the heat shock response has contributed to the advance of biological science, it should be pointed out that most studies focused largely on cellular responses to severe heat stress. Acute exposure to severe heat stress leads to a transient arrest of cell cycle mainly at two checkpoints, the G1/S and G2/M transitions [47–52]. More severe heat stress also leads to programmed cell death, known as apoptosis [53]. However, body or tissue temperature increases by only 1–2 °C during febrile diseases. Therefore, cellular response to mild heat stress is likely to be more important in a physiological sense than that to severe heat stress. As expected, mild heat stress induces HSPs to a lesser extent than severe heat stress does, because the cytoprotective function of HSPs is not demanded [1, 2]. Treatment of cycloheximide, an inhibitor of protein synthesis, blocks HSF1 activation, and HSP expression in HeLa and Rat-2 cells in response to mild heat stress (39–42 °C, 15–20 min), whereas the treatment does not affect the heat shock response by severe heat stress (43–45 °C, 15–20 min) [36, 54]. In addition, cycloheximide also inhibits HSF1 activation due to L-azetidine-2-carboxylic acid, a denaturant of nascent peptides. Thus, mild heat stress seems to affect only newly synthesized polypeptides, resulting in partial HSF1 activation, whereas severe heat stress causes unfolding of pre-existing proteins as well as misfolding of nascent polypeptides, leading to complete activation of HSF1. Despite partial HSP induction, fever-range mild hyperthermia may be beneficial to organisms, although its molecular mechanism is not clearly understood. For instance, fever-range elevation of temperature is presumed to positively regulate cell growth and development, in contrast to severe heat stress [55, 56]. In addition, fever seems to provoke effective immune response through facilitating T cell proliferation and activation [57–59]. Mild heat stress may regulate cell survival through triggering a complex cascade of signaling events, including Ras, Rac1, mitogen-activated protein kinase (MAPK), and other pro-survival molecules that are independent of HSF1-HSP induction [54, 60]. We collected and analyzed the consequences of heat stress to outline the data that support a positive role(s) of mild heat stress in helping cellular events. This review deals with cellular responses to mild heat stress in mammalian cells. First, we summarize the heat-sensing machinery, including cellular proteins and membrane components and their thermodynamic properties.

Heat-sensing machineries

Heat is rapidly sensed by physico-chemical perturbations of various biomolecules in plasma membrane, cytosol and subcellular organelles of cells, which provoke particular signals for the heat shock response. The thermodynamics of cellular constituents are useful for understanding the primary effect of hyperthermia: the structures, activities and interactions of macromolecules reflect their ambient temperature.

Thermodynamics of biomolecules and heat sensing

Heat capacity, the amount of heat involved in the temperature elevation of 1 g of substrate (e.g. protein) by 1 °C, is determined by both the intrinsic temperature dependence of the solvation enthalpy of the protein group and the contribution from the temperature dependence of the protein conformational distribution, which varies with molecules, structures and interactions [61]. Protein structure transition and protein unfolding result in heat capacity change. For example, the enthalpy of the helix-coil transition decreases with the increase of temperature. The values for heat capacity change of the helix-coil transition are found to be negative, which is in contrast to the positive heat capacity change for protein unfolding [62]. The rise in temperature increases the exposure of polar groups in the denatured state and thus decreases the solvation enthalpy difference between the denatured and native conformation. The unfolding enthalpy is decomposed into intraprotein-bonded, van der Waals and electrostatic terms and solvation terms [63, 64]. The denatured state is relatively compact but more labile than the native state, so that the thermal component of heat stress breaks interactions in denatured state more easily than in the native state. This implies that the enthalpy of the denatured state increases with temperature more than that of the native state and contributes significantly to change in heat capacity. In cells, pre-folded nascent polypeptides are more sensitive to heat stress than fully folded proteins: mild heat stress affects only polypeptides being newly synthesized [36, 54]. Three major terms, (i) the primary or covalent structure, (ii) noncovalent interactions arising from secondary and tertiary structure of a protein, and (iii) hydration account for the absolute heat capacity of a protein [65]. For a typical globular protein in solution at 25 °C, the heat cpacity is determined mostly by the covalent structure term (close to 85% of the total) and to a lesser extent by the hydration term (15%). Upon protein unfolding, contribution of the hydration term increases to 40% of the total heat capacity of the protein [65]. Thus, the change in heat capacity upon unfolding is primarily given by the increase in the hydration term.

Macromolecules, in particular polypeptides, have differential sensitivity to heat stress due to their specific heat

capacity. Thermal change determines the structures and interactions of a number of cellular proteins, including surface receptor tyrosine kinases and cytosolic enzymes such as pyruvate carboxylase, to alter their activities [66, 67]. HSF1 has also been demonstrated to directly sense heat stress in vitro and in vivo to be activated in a reversible manner. The sensing of heat stress requires two cysteine residues (C35 and C105), localized within or nearby the HSF1 DNA binding domain, that are required for disulfide bond formation and HSF1 activation in response to heat stress [41–45]. In addition, ion channel activities reflect the temperature applied [68–71]. Activities of ion channels such as Na^+/K^+ and Ca^{2+} flux of mammals are highly sensitive to the change in ambient temperatures and thus suggested to be a kind of thermoreceptor or thermosensor that is involved in the perception of temperature [70]. Macromolecular behaviors such as protein interactions are also determined according to thermodynamics [72, 73]. A typical thermodynamic property of protein-protein interactions has been reported with Ras superfamily proteins, Ras, Rap, TC21 and R-Ras, that play crucial roles in growth factor-mediated cell regulation through interacting with various effector molecules such as the Raf and Ral/guanine nucleotide dissociation stimulator (GDS) [72]. Recognition of multiple effectors is important for communicating signals different ways. Upon association, Ras/effector interactions exhibit a decrease in heat capacity that is one of the most meaningful thermodynamic parameters in terms of characterizing the nature of protein/protein interactions. Although changes in enthalpy, entropy and heat capacity of association with various Ras proteins are similar for the same effector, the thermodynamics of the Ras/Raf and Ras/RalGDS interactions are quite different, indicating that the effectors exert different Ras affinities and that Ras interaction with its effectors is dependent on temperature [72].

Biomembrane fluidity

Biomembranes, including plasma membrane and subcellular organelle membranes, reflect physico-chemical properties of ambient temperature to provoke suitable intracellular signal transduction cascades and to regulate gene expression [74, 75]. Exposing cells to hyperthermic stress disturbs the membrane physical state: membrane lipids primarily undergo a rapid decrease in molecular order to hyperfluidity and form transient local non-bilayer lipid structures such as hexagonal phase. Additionally, lipid-protein interactions in the plasma membrane are also influenced by rapid thermal changes [76]. One phenomenon associated with the acclimation of organisms to changes in ambient temperature is to regulate the fluidity of membrane lipids via changes in the extent of unsaturation of their fatty acids. Desaturase encoded by the *desA* gene introduces specific double bonds and therefore activation of its transcription results in an increase in with membrane fluidity of plasma membrane as well as thylakoid membrane of cyanobacterium *Synechocystis* [77]. HSPs such as GroEL chaperonin and small HSP (alpha-crystallin and *Synechocystis* HSP17) are associated with model lipid membranes and have stabilizing effects on membranes formed of synthetic and cyanobacterial lipids in the bilayer liquid-crystalline state, suggesting that HSPs can modulate membrane lipid polymorphism [78, 79]. Binding is apparently governed by lipid composition and the extent of lipid unsaturation.

Physical alterations in membrane lipids may evoke numerous physiological events: increase in ion fluxes such as Ca^{2+}, Na^+, K^+, H^+, loss of membrane integrity and the change in gene expression. For instance, transition in mitochondrial membrane order by thermal stress is concurrent with temperature-induced alteration in proton leak conductance or thermogenesis [66]. In addition, the influx of extracellular Ca^{2+} stimulates activity of calmodulin-dependent protein kinases, inositol triphosphate production and other signal cascades. Alterations in activity of the Na^+/H^+ exchanger and Na^+, K^+-ATPase result in hyperpolarized membrane potential.

Responses of the cellular membrane to temperature shift depend largely on the molecular characteristics of lipids such as the degree of saturation and the length of membrane lipids. The membrane's physical properties appear to be related to induction of HSPs [75, 80, 81]. Membrane lipid perturbation modifies the set point of the temperature of heat shock response in yeast [80, 81]. Addition of a saturated fatty acid (SFA) induces a strong increase in heat shock messenges RNA (mRNA) transcription when cells are heat-stressed, whereas treatment with an unsaturated fatty acid (UFA) reduces or eliminates the level of heat shock gene transcription at 37 °C. In addition, short chain fatty acids including butyric and propionic acids suppress the expression of HSPs in response to heat stress. Furthermore, HSP coinducers such as bimoclomol with no effect on protein denaturation specifically modulate the membrane lipid phase, indicating that perturbation of the lipid phase is sensed and transduced into a cellular signal, leading to enhanced activation of heat shock genes [82].

Definition of mild heat stress

Before discussing the cellular response to mild heat stress, it is important to distinguish between mild (natural and usually beneficial) and severe (mostly destructive to physiological events) heat stress. However, it is very difficult to define the terms 'mild' and 'severe', since the effects of heat stress are determined by both heat temperature and exposure time: as temperature increases by 1 °C, the time required for the same extent of heat shock re-

sponse is reduced twofold [83, 84]. Furthermore, heat shock sensitivity varies depending on biological factors, including cell types, tissue origin, developmental stage, and cell cycle phase of the cell line analyzed and the cellular events measured. Thus, the criteria for grading heat stress should be considered in both theis arithmetic and biological aspects. Here, we propose criteria for distinguishing between mild and severe heat stress: the criteria are based on the effects of mild versus severe stress on several experimental parameters such as protein denaturation, HSF1 activation/HSP synthesis, cell cycle, cell growth and differentiation, apoptosis, acquisition of thermotolerance and activation of signaling pathways (table 1). The major difference between the cellular responses to mild and severe heat stress is adaptation of growth conditions (mild) versus cell death or morbidity (severe).

Table 1. Criteria for distinguishing between mild and severe heat stress.

	Mild heat stress	Severe heat stress
Denaturation of		
nascent polypeptides	+	+
pre-existing proteins	–	+
HSF1 activation	+ (Rac1 dependent)	++ (Rac1 independent)
HSP synthesis	+ (Rac1 dependent)	++ (Rac1 independent)
Cell cycle arrest		
p21	–	G1/S and G2/M arrest
Cell proliferation	+/– (depending on cell types)	–
cyclin D1	+	↓
cyclin A	+	–
Differentiation	+/– (depending on cell types)	–
Apoptosis	–	+
Acquisition of thermotolerance	+	+
Signaling pathways		
Ras/Rac 1	+	–
PI3K-AKT	+	–
ERK1/2	+	+
SAPK/JNK	+	+
p38MAPK	+	+
Biological relevance	adaptation of growth conditions	cell death or cell morbidity

Regulation of cell survival pathways by mild heat stress

Whereas severe heat stress has been shown to lead to cell cycle arrest and apoptosis [47–53], mild heat stress is presumed to positively regulate cell cycle progression and differentiation, through multiple Ras signal pathways involving the Raf-extracellular-regulated kinase 1/2 (ERK1/2) pathway, phosphatidylinositol-3 kinase (PI3K)-Akt/PKB-glycogen synthase kinase (GSK)-3β pathway, and Rho-Rac1-nicotinamide adenine dinucleotide phosphate (NADPH) oxidase pathway (fig. 1). First, we discuss the regulation of these pro-survival signal pathways by mild heat stress.

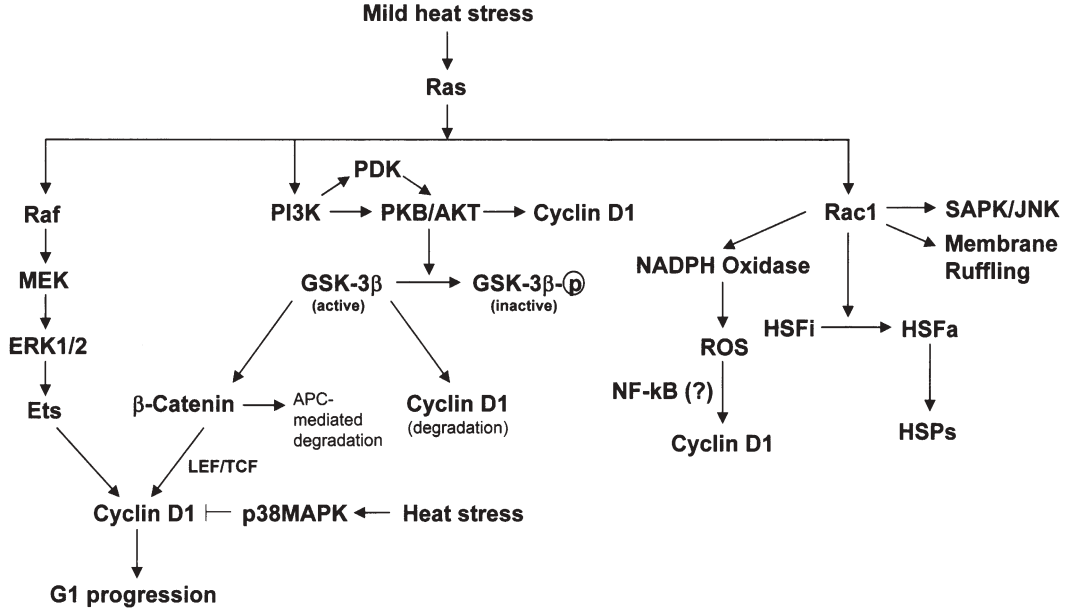

Figure 1. The signal pathways responsible for cyclin D1 synthesis and HSF1 activation/HSP expression in response to mild heat stress in mammalian cells. HSFi, inactive HSF1; HSFa, active HSF1.

Ras and Rac1

Ras superfamily GTPases are membrane-bound small GTP-binding proteins that play a crucial role(s) in diverse cell physiology, including cell cycle progression, cell division, regulation of cell morphology and motility, and intracellular trafficking of molecules and organelles [85–95]. Ras functions as a relay switch that is positioned downstream of cell surface receptor tyrosine kinases (RTKs) and upstream of a cytoplasmic signal cascade. The biological activity of Ras proteins is mediated by multiple signaling pathways, including the Raf-ERK1/2 pathway, PI3K-Akt/PKB-GSK-3β pathway and Rho-Rac1-NADPH oxidase pathway. Rho GTPases of the Ras superfamily are 20–30-kDa GTP-binding proteins and include RhoA, B, C, D, E; Rac1, 2; and Cdc42. Rho GTPases also play a wide range of physiological roles in actin cytoskeleton regulation, transcriptional regulation, growth and development [85–97].

Mild heat stress (39–42 °C, 15–20 min) has been shown to activate Ras and Rac1, major components of Ras signaling pathways, as demonstrated by pull-down assay using the Ras binding domain of Raf and the Rac1 binding domain of p21-activated kinase (PAK), respectively [54, 60]. In addition, mild heat stress induces membrane ruffling in a Rac1-dependent manner, similar to that observed in growth factor-treated cells [54], whereas severe heat stress (44–45 °C, 15–20 min) causes breakdown of actin stress fiber [98–100]. It is not clear how mild heat stress activates Ras and Rac1 GTPases. Although heat stress induces the release of fibroblast growth factor (FGF)-1 from NIH3T3 cells [101], Ras and Rac1 activation by mild heat stress is not likely mediated by heat stress-secreted growth factor since the activation occurs rapidly (within 5 min after mild heat shock treatment) [54, 60]. Mild heat stress may activate multiple growth factor receptors, including epidermal growth factor (EGF) receptor tyrosine kinase, by affecting membrane structure and mobility, which in turn activates the Ras signal pathway [102]. Otherwise, the Ras molecule may be directly activated by heat stress [72].

As Ras and Rac1 regulate a huge number of cellular processes including cell cycle, transformation, cell migration and thermosensitivity, through multiple cooperating pathways, the consequences of Ras and Rac1 activation by mild heat stress may be diverse. One of them may be HSF1 regulation [54]. Overexpression of Rac1N17, a dominant negative mutant Rac1, completely prevents HSF1 activation and HSP expression in response to mild or moderate heat stress (40–43 °C, 20 min), but not to severe heat stress (44–45 °C, 20 min) [54]. Similarly, Rac1N17 inhibits HSF1 activation and HSP expression by hypoxia/reoxygenation and sodium arsenite [103] and by mechanical stress in vascular smooth muscle cells [104], indicating that the Rac1 GTPases play a critical role(s) in stress-induced HSF1 activation and HSP70 expression (fig. 1). However, constitutively active Rac1V12 does not induce HSF1 activation, suggesting that Rac1 may be necessary but insufficient for HSF1 activation [54, 103]. Although reactive oxygen species (ROS) participate in HSF1 activation by hypoxia/reoxygenation and sodium arsenite [103], they are is not likely involved in heat stress-induced HSF1 activation and HSP expression, as diphenyleneiodonium, an NADPH oxidase inhibitor, and other antioxidants such as pyrrolidine dithiocarbamate, butylated hydroxytoluene and ascorbic acid do not exert inhibitory effects on heat stress-induced HSP regulation [54]. Since mild heat stress affects only newly synthesized polypeptides [36, 54], the Rac1 GTPase signal pathway may be implicated in the molecular mechanism that specifically recognizes misfolding of nascent polypeptide, but not of formerly folded proteins to activate HSF1. Thus, the efficiency of protein synthesis and folding occurring on the ribosome may be communicated to the cytoplasm via several signal transduction pathways, including Rac1 signaling.

PI3K and Akt/PKB

PI3K is one of the most important regulatory proteins involved in cell functions such as mitogenic signaling, growth and survival, cytoskeletal remodeling, metabolic control and vesicular trafficking [105, 106]. PI3K can be activated via Ras in response to growth factor stimulation and possess double-enzymatic activity, lipid kinase and protein kinase. Among the main effectors of PI3K are the mitogen-transducing signal proteins such as phosphoinositide-dependent kinases (PDKs), protein kinase C (PKC), and MAPK. In most case, the anti-apoptotic effect of PI3K is mediated by a serine/threonine kinase Akt/PKB that is the best-characterized pro-survival protein kinase [105–113] and is constitutively activated in many cancer cells [113–116]. The pro-survival kinase, can also be activated via the PI3K-independent pathway, including protein kinase A (PKA) or calmodulin-dependent protein kinase kinase [105, 106]. Akt/PKB regulates cell viability through both activating pro-survival molecules and inactivating pro-apoptotic molecules. For example, it phosphorylates and inactivates GSK-3β, which plays important roles in apoptosis, cell cycle regulation and insulin-mediated glycogen metabolism [105, 106, 108, 109]. In addition, Akt/PKB inhibits the pro-apoptotic protein Bax conformational change that is responsible for apoptosis in response to various stresses [113].

Moderate heat stress (43 °C) has been shown to induce the maximal increase of c-Src activity that recruits PI3K to the Src homology (SH)-2 domain to activate PI3K in NIH 3T3 fibroblasts [102]. Additionally, mild heat stress (41–42 °C) induces phosphorylation and activation of Akt/PKB in a PI3K-dependent manner in NIH 3T3 fibroblasts [60, 117], whereas prolonged treatment of

severe heat stress (44–45 °C) fails to activate Akt/PKB. Mild heat stress also inactivates GSK-3β through Akt/PKB phosphorylation at Ser-9 [60]. LY294002, a PI3K inhibitor, significantly blocks phosphorylation of Akt/PKB and GSK-3β in response to heat stress, suggesting heat shock activation of the PI3K-Akt/PKB-GSK-3β pathway. In vivo experiments show that mild heat stress (41 °C, 30 min) application induces PI3K activation and GSK-3β inactivation in the absence of poly (ADP-ribose) polymerase (PARP) degradation, a sign of apoptosis [118]. Akt/PKB and GSK-3β phosphorylation by heat stress is transient (detected only in cells heat stressed at 41–43 °C for 40 min, but not in cells recovered at 37 °C) compared to that induced by growth factors [60]. Mild heat stress-induced PI3K-Akt/PKB activation may be associated with the apoptosis-suppressive effect of mild heat stress, in contrast to severe heat stress.

MAPKs

Among the many signaling pathways that respond to mitogens and stresses, MAPK family members are crucial for maintenance of cells through regulating the activities of nuclear transcription factors. [119–121]. Three subfamilies of MAPKs have been identified: ERKs, SAPK/JNKs and p38MAPKs. It was originally shown that ERKs are important for cell survival, whereas SAPK/JNKs and p38MAPKs are more strongly tied to stress and thus involved in apoptosis [122–125]. It is accepted that the balance between the magnitude of ERK and SAPK/JNK/p38MAPK activation is key to determining whether the cells survive or undergo apoptosis. However, many investigators have recently demonstrated that regulation of apoptosis by MAPKs is more complex than initially thought and often controversial [124–127]. For instance, SAPK/JNK activation potentially promotes or inhibits apoptosis depending on the cell type and the types and strength of stresses.

Instant or mild heat stress (43 °C, 5 min) activates ERK1/2 through its phosphorylation at Thr-202/Tyr-204 in NIH 3T3 fibroblasts, which can be blocked by the dominant negative Raf mutant and partially by wortmannin [102]. Mild heat stress (42 °C)-induced ERK1/2 activation is transient, which is different from serum-stimulated persistent ERK1/2 induction [60]. Mild heat stress also induces ERK1/2 transiently in the cerebellum in vivo but does not induce it at all in rat liver and hippocampus in vivo [118, 128]. Mild heat stress appears to stimulate SAPK/JNK in a Rac1-dependent manner, whereas severe heat stress-mediated SAPK/JNK activation is regulated independently of Rac1. Heat stress induces phosphorylation of p38MAPK at Thr-180/Tyr-182, while serum addition does not [60]. In rat liver in vivo, mild heat stress (41 °C, 30 min) promotes activation of SAPK/JNK and p38MAPK and their upstream kinases such as MKK3/6 and PAK but does not affect glutathione S-transferase (GST) and germinal center kinase (GCK) [118]. p38MAPK can phosphorylate HSP27 which dissociates into monomer or dimmer, resulting in stabilization of microfilaments to protect cells from deleterious signals [129]. Mostly, p38MAPK is known to play a role(s) in triggering the apoptotic process in response to various stresses. In addition, p38MAPK activated by a low dosage of oxidative stress is involved in mitotic arrest [130]. Even brief heat stress (44 °C, 10 min) as well as oxidative stress activates apoptosis signal-regulating kinase-1 (Ask1) that stimulates the activity of p38MAPK and SAPK/JNK [131]. Ask1, which activated via dissociating from its inhibitors, GSTM1-1 (glutathione S-transferase Mu1-1), for heat stress or redox-sensing protein, thioredoxin, for oxidative stress. At the present time, the consequences of ERK1/2, SAPK/JNK and p38MAPK activation by heat stress are not clear since their acting mechanism is very complicated and diverse [122–127].

Cyclin D, a mediator of cell cycle regulation

Cell cycle is finely controlled by the cooperation of multiple Ras effectors [132–136]. When quiescent cells enter the cell cycle in response to mitogenic signals, they induce genes encoding D-type cyclins (D1, D2 and D3), key molecules required for passage through the restriction point in the mammalian cell cycle [132, 133, 137]. The cyclins assemble with their catalytic partners, cyclin-dependent kinase (CDK) 4 and CDK6, as cells progress through G1 phase, thereby inactivating the growth-suppressive function of retinoblastoma (Rb) protein through its phosphorylation [132, 133, 138]. Cyclin D-CDK4/6 complex also titrates CDK inhibitors, such as $p27^{Kip1}$ and $p21^{Cip1}$, facilitating cell cycle progression [132, 133, 135, 139]. Growth factor-induced cyclin D1 expression is mainly regulated by multiple Ras signal pathways which involve (i) the Raf/MAPK kinase (MAPKK)/ERK1/2 pathway, (ii) the PI3K/Akt/PKB pathway and (iii) the Rac1/NADPH oxidase/ROS pathway [132, 133, 140, 141]. The level of cyclin D1 is also post-transcriptionally controlled by the PI3K-Akt/PKB pathway [132, 133, 136]. GSK-3β, a downstream effector of Akt/PKB, can phosphorylate cyclin D1 and thereby stimulate its nuclear export and accelerate its ubiquitin-dependent proteasomal degradation in the cytoplasm. Furthermore, GSK-3β is involved in targeting the adenomatous polyposis coli (APC)-mediated degradation of β-catenin, which regulates the expression of cyclin D1. p38MAPK, which is induced by several different kinds of stresses including heat stress, has also been implicated in downregulation of cyclin D1 by regulating its transcription and degradation [132, 133].

The effects of heat stress on the cell cycle depend on the strength and the duration of applied heat stress [48, 142, 143]. Acute exposure to heat stress leads to a transient arrest of cells at mainly two cell cycle check points, the G1/S and G2/M transitions, through inducing $p21^{WAF1}$ CDK inhibitor and other regulatory proteins [47–52]. In addition, severe heat stress reduces the level of cell cycle regulatory molecules, including cyclin D1, CDK and phosphorylated Rb, resulting in a transient cell cycle arrest mostly at the G1 stage of the cell cycle [47–50]. If once HSPs are induced, thermotolerance is acquired and normal cell cycle resumes [47]. Severe heat stress also suppresses the hyperphosphorylation of Rb via the p53-induced CDK inhibitor p21, which prevents S phase entry in EGF-stimulated Swiss mouse 3T3 cells [47].

Recently, it was demonstrated that exposing quiescent serum-starved NIH3T3, Rat-2, and HeLa cells to heat stress conditions in a range of 39–43 °C increases the cyclin D1 level in a temperature- and time-dependent manner [60]. While serum stimulation of quiescent cells maintains the increased levels of cyclin D1, heat stress transiently increases its levels, with the maximal induction at 9 h after heat stress at 42 °C for 40 min and at 16 h after prolonged exposure to 39.5 °C. Mild heat stress also causes cyclin D1 to assemble with CDK4/6 and to translocate to nucleus. Although heat stress has been shown to induce secretion of growth factors such as FGF in animal cell cultures even though in an inactive form [101], heat stress itself acts as a signal activator for the cyclin D1 induction. Mild heat stress-induced cyclin D1 expression is mediated through multiple Ras signal pathways involving ERK1/2, PI3K/Akt (PKB)/GSK-3β and Rac1/NADPH oxidase, which are responsible for growth factor-induced cyclin D1 expression [60] (fig. 1). While mild heat stress induction of cyclin D1 is regulated mostly at the transcriptional and translational levels, its turnover is also controlled by heat stress. Since heat stress induces the phosphorylation and inactivation of GSK-3β, which stimulates degradation of cyclin D1 as well as β-catenin, which regulates the expression of cyclin D1, cyclin D1 degradation could be inhibited by heat stress. Transient ERK1/2 activation and GSK-3β inactivation may explain this transient cyclin D1 induction in response to heat stress. p38MAPK may also be involved in this phenomenon.

When cells are exposed to prolonged heat stress at 39.5 °C, cyclin D1 induction is sustained for a longer period (until 16 h after heat stress). In addition, prolonged exposure to mild heat stress is sufficient for induction of cyclin A, which is required for DNA synthesis and begins to be synthesized as cells approach the G1-S transition [132–136]. However, in NIH3T3 and Rat-2 cells, mild heat stress itself does not exert significant stimulatory effects on cell proliferation. Mild heat stress may facilitate growth factor-stimulated cell proliferation through inducing cyclin D1. In fact, fever-range hyperthermia is known to facilitate interleukin 1-dependent T cell proliferation and activation [58]. It was recently demonstrated that mild heat stress stimulates cell proliferation and DNA synthesis in human bone marrow stromal cells and MG-63 cells in vitro [144]. Thus, the effect of mild heat stress on cell cycle progression or proliferation is quite ambiguous, and cellular response to mild heat stress varies according to types of cell line analyzed.

Differentiation and immune response regulation

Information about fever-range hyperthermia has been focused on immune response since immune systems respond to thermal change sensitively and differentially. Febrile rise in core body temperature is shown to enhance innate and adaptive immunity through positively regulating cell growth and differentiation [57–59]. Fever-range hyperthermia enhances interleukin (IL)-1-dependent T cell proliferation and activation [58] and granulocyte-macrophage colony-stimulating factor-induced differentiation of human leukemia cells U937 [145]. Treatment with heat stress (41.8 °C, 60 min) has been used as an adjunct to chemotherapy in patients with various malignant diseases. The treatment induces prolonged T cell activation in the patients, blood: a drastic increase in peripheral natural killer cells and $CD56^{+}$-cytotoxic T lymphocytes, a marked but short-lived increase in IL-6 and an increase in the percentage of peripheral cytotoxic T lymphocytes expressing CD56 [146]. Heat stress sensitivity of immune response depends on the developmental stage of the cell. For instance, embryonic thymocytes are able to survive and differentiate normally in response to heat stress, whereas adult thymocytes rapidly undergo apoptotic cell death [147]. The levels of the HSPs may be responsible for in vivo immune cell regulation, since embryonic thymocytes, but not mature thymocytes, are able to synthesize HSP68 continually for up to 4 h under hyperthermia [147]. Fever-like mild heat stress also functions as a positive regulator of terminal differentiation in several other cell types, such as human myeloid leukemic HL-60 cells, neuroblastoma (N1E 115) cells, thyroid carcinoma cells and chondrosarcoma [148–152]. However, the molecular mechanism of HSP-mediated cell differentiation is not clearly understood [147, 151, 152]. Since mild heat stress partially activates HSF1 and induces HSP expression to a lesser extent than severe heat stress [1, 2, 36, 54], mechanisms other than HSPs may also be involved in immune cell regulation by mild heat stress. In fact, mild or moderate hyperthermia enhances, independent of HSF1 activation, maturation of immune cells, e.g. dendritic cells, which play a major role in innate and adaptive immunity [153]. Mild heat stress also induces spectrin reorganiza-

tion via activation of PKC within T lymphocytes, which is observed during T lymphocyte activation [154, 155]. Thus, a thermal element of fever can modulate critical steps in signal transduction pathways necessary for effective lymphocyte activation and function.

When activated by antigens, helper T (Th) cells differentiate into one of several subtypes, characterized by their distinct cytokine production patterns. Th1 cells are known to activate cellular immunity, resulting in inflammatory response, whereas Th2 cells induce humoral and allergic responses and suppress inflammation. Th1 and Th2 effector functions and their development are attributable to their distinct cytokine expression patterns. The ability to selectively produce Th1-cytokine [e.g. IL-2, interferon (IFN)-γ, tumor necrosis factor (TNF)-α] and Th2-cytokine (e.g. IL-5 and IL-13) and thereby to regulate Th1/Th2 cytokine balance is highly temperature dependent and tissue specific [59, 156]. For example, when staphylococcal enterotoxin B (SEB)-stimulated whole blood is incubated at 38–42 °C, it favors Th2 cytokine production to alter Th1/Th2 cytokine balance [59, 156], whereas in situ heated-prostate cancer cells favor Th1-cytokine release of tumor-infiltrating T lymphocytes [157]. However, lipopolysaccharide (LPS)-induced expression of IL-18, an important cytokine that has diverse immune regulatory effects on T, B, natural killer cells and non-immune cells, is significantly suppressed by heat stress in murine peritoneal macrophages [158]. Activation of helper T cells mediated by the T cell receptor induces a series of biochemical events. For example, Ras-, PKC- and calmodulin/calcineurin-mediated pathways play a central role in signal transduction of cytokine gene expression, and a balance between the signaling pathways contributes to Th1/Th2 cytokine production. Mild heat stress-induced survival signal pathways may be linked to effective lymphocyte activation and cytokine secretion.

Febrile temperature rapidly promotes neutrophil migration [159] and secretion of antibacterial chemicals [160, 161]. In addition, fever-range hyperthermia (40 °C, 6–12 h) augments actin polymerization in vascular endothelial cells and enhances the ability of endothelial-derived factors to transactivate the $\alpha 4\beta 7$ integrin lymphocyte homing receptor [162]. In contrast, mild heat stress does not affect expression of adhesion molecules (ICAM-1, E-selectin, VCAM-1, P-selectin, PECAM-1, PNAd, MAdCAM-1), cytokine release (IL-1β, TNF-α, IFN-γ, IL-6, IL-11, IL-12, IL-13) or chemokine secretion (IL-8, RANTES, MCP-1, MIP-1β, MIG) in endothelial cells [163]. This implies that hyperthermia avoids unproductive exodus of lymphocytes to non-involved extralymphoid tissues while simultaneously promoting lymphocyte delivery to sites of immune activation. The signal pathways for this cell regulation are not clearly understood, but mild heat stress-induced cell signal pathway may be responsible for this event.

Thermotolerance

It is well known that preconditioning mild heat stress causes a transient and cross-resistance of cells and various organs such as lungs, myocardium and kidney to a second heat challenge and other environmental stresses, which is termed acquired thermotolerance [164–166]. For instance, mild heat shock preconditioning induces protection against neurotoxicity of 1-methyl-4-phenylpyridinium, a neurotoxin that selectively targets dopaminergic cells [167]. The molecular mechanism(s) for the development of thermotolerance has not been clearly understood. Thermotolerance development and decay are dependent on the cell cycle phase [168, 169]: thermotolerance develops faster in G1 than in G2/M phase cells, whereas it does not at all in S phase cells. Thermotolerance is decayed accompanying with the entry of formerly G1-arrested cells into S phase. Although many cells, particularly S phase cells, die from the chromosomal aberration, some cells are apt to develop thermotolerance or adaptation.

The HSPs appear to play a critical role in the development of thermotolerance and protection from cellular damage associated with various stress stimuli such as ischemia, cytokines, energy depletion and oxidative stress [164–166, 170–173]. Particularly, HSP70 has been shown to play a critical role in cell survival and thermotolerance in response to stresses, possibly through inhibiting a number of anti-survival pathways such as the SAPK/JNK pathway [174–179]. Disruption of the double-stranded RNA-dependent protein kinase gene that was recently identified as essential for efficient activation of the heat shock response through stabilization of HSP70 mRNA species blocks the development of thermotolerance [179].

However, some cells such as HL-60 cells are able to induce thermotolerance even when they fail to express HSP70, suggesting that HSP70 expression is not obligatory in thermotolerance induction. In the yeast *Saccharomyces cerevisiae*, mild heat treatment strongly induces HSP104, which provides acquisition of thermotolerance. HSP104 plays a crucial role in keeping cells from being damaged by oxidative stress, thus acting as a modulator of the intracellular redox state [180–184]. HSP27 has also been implicated in acquired thermotolerance [185–187]. Human cells infected with virus such as mumps virus were recently shown to be more susceptible to apoptosis caused by extracellular stresses. The susceptibility is due to suppression of HSP27-dependent thermotolerance by the viral accessory protein V-mediated destruction of STAT-1 [188]. STAT-1 is required for transcriptional activation of the HSP27 gene, but not for the HSP70 gene, in addition to the activated HSF1 [188]. HSP70 and HSP27 have also been implicated in translational thermotolerance; heat stress results in inhibition of general protein

synthesis and in thermotolerant cells, protein synthesis is still rapidly inhibited by heat stress, but recovers faster than in naive heat-shocked cells, a phenomenon known as translational thermotolerance. Overexpression of HSP27 protects cap-dependent initiation of translation, while HSP70 overexpression protects both cap-dependent and -independent translation, indicating that translational thermotolerance would be a co-operative effect of different heat shock proteins [189].

HSF1 is also critical for maintaining cellular integrity after heat stress, cells from hsf1–/– mice lack the ability to develop thermotolerance [190–191]. This deficiency is explained by the elimination of stress-inducible HSP70 and HSP27 response in the absence of HSF1 activity, leading to a lack of HSP-mediated inhibition of apoptotic cell death via both caspase-dependent and caspase-independent pathways [191]. However, coinfection with adenoviral HSP70 and HSP27 constructs did not fully recreate thermotolerance in either hsf1+/+ or hsf1–/– mouse embryo fibroblasts (MEFs), indicating that other HSF1-mediated gene expression is required for complete thermotolerance, and proteins other than HSP70 and HSP27 are also implicated in thermotolerance [190].

The disaccharide trehalose, which accumulates dramatically during heat shock and stationary phase in yeast, enhances thermotolerance and reduces aggregation of denatured proteins [192, 193]. Trehalose accumulation decreased the initial appearance of damaged proteins, presumably by acting as a free radical scavenger. Therefore, trehalose accumulation in stressed cells plays a major role in protecting cellular constituents from oxidative damage.

In addition to the HSPs, HSF1 and trehalose, pro-survival signal molecules may also be involved in thermotolerance. As described previously, mild heat stress triggers a complex cascade of signaling events, including Ras, Rac1, MAPK and other pro-survival molecules, which may be responsible for the development of thermotolerance [54, 60, 102, 117]. Further studies on this powerful protective adaptation of cells may contribute to a better understanding of the cellular responses to mild heat stress and to the design of cytoprotective pharmacological agents.

Conclusion

Although a number of investigators have demonstrated that severe heat stress exerts cytotoxic effects on organisms, including induction of apoptosis and cell cycle arrest, fever-range elevation of temperature or mild heat stress may be beneficial to living cells through positively regulating cell proliferation and differentiation. Although body or tissue temperature increases by only 1–2 °C during febrile diseases, it is sufficient to produce multiple changes that ultimately affect both the structure and function of several proteins and membrane fluidity. The change in the fluidity of membrane lipids may be the first event that signals a change in temperature, and thus is regarded to act as a thermosensor. Fever-range hyperthermia- or mild heat stress-induced increase in membrane fluidity may result in activation of several membrane proteins, including growth factor receptors, which in turn activate intracellular signal transduction cascades such as the Ras signal pathway. Disturbance of the membrane physical state by heat stress may cause transduction of a signal that induces the heat shock response, such as HSF1 activation and HSP expression. In this respect, we suggest that mild heat stress may act as one of physico-chemical signals that may play a critical role(s) in cell growth and differentiation through modulating the physical properties and activities of several important regulatory proteins. Although mild heat stress itself is not sufficient for cell growth and differentiation, it may facilitate growth factor-mediated cell growth and differentiation. Similar activity in facilitating the action of growth factors is observed with ROS that modulate the structure and activity of biomolecules, in particular proteins, via oxidizing specific redox-sensitive sulfhydryl groups. We further suspect that mild heat stress and ROS may have evolved as primitive signal molecules at an early stage of evolution before the growth factor-mediated cell growth machinery was established. Further careful studies may provide new insights into the elucidation of molecular mechanisms for fever-dependent cell regulation in organisms.

Acknowledgments. This work was supported by a Korea Research Foundation grant (042-E00012).

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