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Review

Heat shock effects on cell cycle progression

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Abstract. In mammalian cells, short-term (acute) exposure to a moderate heat shock leads to a transient arrest of cells at mainly two cell cycle checkpoints, the G1/S and G2/M transitions. This is documented by the more or less synchronous resumption of cell cycle progression from these checkpoints during recovery. The reason for the accumulation of cells at these checkpoints may be found in activity thresholds of cyclin-dependent kinases (Cdks) at both transitions which are determined by (i) the amounts of the responsible cyclins, (ii) regulatory phosphorylation of the Cdks and (iii) the inhibition of Cdks by associated regulatory proteins (Ckis). All three regulatory systems may be subject to heat-shock-dependent changes, the amounts of Ckis, in particular, being increased. Cdk-dependent phosphorylation of the retinoblastoma protein and the subsequent release of active S-phase-specific transcription factors E2F/DP are considered as major heat-sensitive steps in cell cycle progression. Furthermore, high acute heat shock and long-term (chronic) heat treatment may lead to cell-type-specific forms of cell death. All types of responses to heat treatment are subject to adaptation after a 'priming' treatment, probably due to higher levels of heat shock proteins.

Key words. Heat shock; cell cycle; arrest; cell death; thermotolerance; cyclin-dependent protein kinases.

Introduction

Heat shock can arrest or delay the cell cycle as was shown originally by Zeuthen [1] in *Tetrahymena*. In fact, the heat-induced arrest of cells in certain cycle phases was used to synchronize randomly proliferating cell populations of *Schizosaccharomyces pombe* [2]. In the meantime, insights into the cell cycle machinery and the cellular responses to heat shock have increased enormously. The complexity of both systems, however, makes a mechanistic explanation of heat shock effects difficult.

This review first provides a brief account of the general effects of heat shock on cellular processes [3] and then summarizes current knowledge of heat shock effects on cell cycle kinetics, i.e., on the temporal sequence of cell cycle phases and phase transitions. This approach has been widely applied since flow cytometric determination of single-cell DNA content allowed the analysis of proliferation kinetics in cell populations. The results of this approach, however, are often difficult to compare, because the effects of heat shock vary depending on the strength and duration of the shock exposure, on the particular cell line analyzed, on the state of acquired thermotolerance, and on the measured cellular event, for example, immediate or delayed arrests at different cell cycle phases, clonogenity, or cell death. We will focus on the different responses in order to derive explanations for sometimes controversial data, and we concentrate on mammalian cells because they are the most well-analyzed group of cells with respect to heat shock effects on the cell cycle, general cell cycle regulation, and heat shock responses.

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Among the most well established heat shock effects on cell cycle kinetics are the transient arrests of cells at the G1/S or G2/M border, which correspond to so-called 'checkpoints' or control processes at these points. We will discuss these arrests in terms of possible mechanistic explanations starting with a brief summary of the most important, possibly temperature-sensitive control processes within the cell cycle. We then go on to summarize the available data at the molecular level with respect to heat shock effects on cell cycle regulators. Even though much important data are lacking, effects of heat shock on cyclin-dependent kinases (Cdks) as well as mitogenic kinase cascades are combined in two models of heat-induced cell cycle arrests in G1 and G2.

Understanding the various pathways by which elevated temperatures affect the cell cycle clock, the timing of cell cycle events, and transient thermotolerance may be of practical importance when applying hyperthermia during cancer therapy [4], since in cancer cells, one or more part(s) of the regulatory network controlling cell cycle progression is defective [reviewed in refs. 5, 6]

General heat shock effects

Heat shock affects almost every process in and many structural components of a living cell, including the cell cycle and its control [3]. One can distinguish direct or primary effects of heat shock and the heat shock response. The direct effects comprise all the physicochemical and molecular changes induced by heat shock, some of which are summarized below, whereas the heat shock response can be defined as the active cellular responses to these changes—often aimed at attenuating the direct effects.

Direct effects

Table 1 gives a brief overview over some direct effects of heat shock which may interfere with the cell cycle processes discussed below. The general inhibitory effects of heat shock on the transcription, processing, and translation of most genes and their products should inhibit the synthesis and accumulation of cell-cycle-specific proteins, particularly cyclins and the expression of S-phase-specific genes and transcription factors, such as E2F. The degradation of proteins by the ubiquitin-proteasome and the lysosome-located pathway is generally enhanced by heat shock. Both effects on gene expression as well as on protein degradation seem to perturb cell cycle progression considerably.

Kinases and phosphatases play important roles in cell cycle progression. In general, both inhibitory and activating effects of heat shock on various kinases and phosphatases and the phosphorylation of proteins have been described. Different responses have also been observed in enzymes involved in the cell cycle.

Heat shock generally enhances membrane permeability and thus increases the flux of ions. The observed increase in intracellular calcium enhances the activity of calcium calmodulin-dependent protein kinases and other signal cascades. Heat shock also increases sodium and proton concentrations. The increased proton permeability of mitochondrial membranes probably contributes to ATP depletion. This depletion—in particular during chronic heat exposure—inhibits ATP-driven processes including the cell cycle. Ionic and ATP changes also contribute to perturbation of the cytoskeleton (microtubules, intermediate fibers, microfilaments). This should particularly inhibit spindle formation and thus block mitosis, as has been observed, but the molecular details are poorly understood [3].

Table 1. Direct effects of heat shock on some cellular processes. The older literature on heat shock effects is reviewed in Nover [3], the general effects of temperature on cellular processes in Alexandrov [28]. In addition, a few comprehensive articles are listed.

Process	Effects	References
Transcription	mainly inhibition (except for heat shock genes)	3, 7
RNA processing	inhibition, especially of splicing	3, 8, 9
Translation	inhibition of initiation factors, ribosomal proteins	3, 10–14
Intracellular distribution of proteins	changes in the amounts of nuclear proteins, particularly heat shock proteins	3, 15, 16
Degradation of proteins	activation of some parts of the ubiquitin-proteasome and lysosomal pathway, inhibition of synthesis of lysosomal proteases	3, 17–20
Activity of enzymes, especially kinases and phosphatases	inhibition or activation	21, 22
Membrane structure and function, ion concentrations	destabilization, increase in permeability, increase of calcium, proton, sodium	23–26,
Energy metabolism	decrease in ATP concentration	27
Cytoskeleton	destruction, depolymerization	3

Converting extracellular stimuli into appropriate intracellular responses involves the coordinate action of specific intracellular pathways. One of the most prominent signal transduction pathways identified in all eukaryotic cells involves the mitogen-activated protein kinase (MAPK) cascades [29]. In mammalian cells, three distinct subgroups of MAPK cascade can be distinguished [30, 31]: the stress-activated MAP kinases SAPK/JNK (stress-activated protein kinase/c-jun terminal kinase), the HOG-1 (high osmolarity glycerol) homolog p38 which are mainly activated upon environmental stress, and the mitogen-activated kinase extracellular signal-regulated kinase (ERK) predominantly activated by growth factors. Dependent on the cellular context, different cellular responses (proliferation, differentiation, and apoptosis) are initiated through the preferential activation of a specific MAPK cascade. It is now well established that members of the mitogen-activated kinase, p44/ERK1 and p42/ERK2, play critical roles in the control of cell cycle progression during meiosis and mitosis, while the activation of SAPK by environmental stress leads to growth arrest or, in some cell types, to apoptosis [32, 33]. Heat shock has also been shown to cause a transient activation of G proteins and an increase in second messengers such as cAMP and inositol 1,4,5-trisphosphate (IP_3) [26].

Heat shock response

Cells counteract the effects of stress by increasing the synthesis of heat shock (stress) proteins (HSPs) [34, 35] which represent an important part of the heat shock response. Many of the stress-induced HSPs, such as HSP90, HSP70, and the small HSPs, are isoforms of the constitutive chaperones and fulfill similar or identical functions. Within cell cycle processes and signal cascades, chaperones play an important stabilizing role: they associate with cell cycle or signal proteins in order to translocate them to their targets, to keep them in a required conformational state, or to eliminate degraded or mutated forms [36]. HSP90, in particular, acts as a dimer and forms complexes with protein kinases, steroid receptors, and transcription factors with the aim of guiding the protein partner to its ultimate location, maturing its conformation, and/or stabilizing an inactive (or active) conformation [37, 38]. The HSP70 family whose members are found both in the cytosol and in various cell compartments is equally or even more involved in the functions described above. The constitutive members of this family (also called HSP70 cognates or HSC70) in the cytosol are particularly involved in folding and refolding of proteins, in maintaining their unfolded (transport) state, and in the elimination of nonfunctional proteins [34]. They do this by cooperating with so-called 'cochaperones,' for example with HSP40, the eukaryotic homologue of DnaJ [39]. They also form complexes with HSP90 and their substrates as well as with cochaperones [40, 41]. Several mitogenic signal or cell cycle control proteins appear to be kept in a certain conformation by such multiprotein complexes [36]. Chaperones of the HSP70 family also help proteins to enter the nucleus and to associate with or dissociate from other nuclear protein molecules [16]. Furthermore, this chaperone family is synthesized in a cell-cycle-dependent manner with highest concentrations within S phase [42–45].

Evidence for a role of HSP70 in cell proliferation was obtained from *hsp70* antisense application, which abolished progression through G1 and S phase in human tumor cells [46]. With the increased amounts of HSPs after heat shock, many cellular functions become thermotolerant [35, 47]; this is true also for the heat-induced arrests of the cell cycle [N. M. Kühl, J. Kunz and L. Rensing, unpublished data] and for apoptosis-inducing treatments [48], supporting the notion of a protective function of chaperones and stress proteins in maintaining cell cycle and signalling processes due to the formation of complexes [36, 49].

Heat shock effects on cell cycle kinetics, differentiation, and cell death

Heat shock effects on the cell cycle depend on the form of heat shock treatment, which may explain some of the discrepancies among published results. Heat shock conditions normally applied to mammalian cells are short term (acute, < 1 h) with temperatures between 40 and 45.5 °C, or long term (chronic, > 1 h) at around 41.5 °C. Choice of heat shock treatment strongly influences the results, and may result in growth stimulation, cell cycle phase and transition delays, cell cycle arrests, or different modes of cell death depending on the severity of the stress [50–53]. Heat shock sensitivity itself is cell type and partially cell cycle phase dependent: the same heat shock treatment may result in different effects when using different cell lines or cells in different cell cycle phases [51, 54]. These response types and the cell cycle regulators that may be involved are briefly described below. Because acquired thermotolerance greatly influences cell cycle progression during chronic hyperthermia, acute and chronic treatments are discussed separately.

Acute (short-term) heat shock treatment

During the early work on heat shock effects on the cell cycle, the only processes directly accessible and shown to be inhibited by heat shock treatment were DNA synthesis and cell division [1, 2]. Following the invention of flow cytometry, major progress was made,

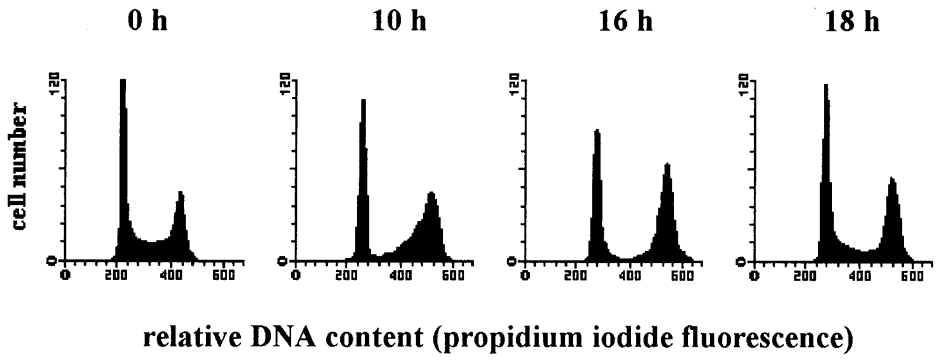

Figure 1. Heat-induced cell cycle phase redistribution. Asynchronously proliferating rat C6 glioma cells (0 h) were heat shocked for 30 min at 44 °C, returned to 37 °C and cultivated for the times indicated. Thereafter, propidium-iodide-stained samples were analyzed by flow cytometry. The relative DNA content of 10,000 cells was plotted against the cell number for each histogram. After 10 h of recovery, no more G1 cells move into S phase due to a G1 block whereas S phase cells resume DNA synthesis and start accumulating in G2. Cell cycle progression of G2/M cells is also inhibited leading to a phase distribution pattern with nearly all cells in the G1 or G2/M fraction 16 h after the heat shock. After 18 h of recovery, G1 cells enter S phase and G2/M cells divide and appear in the G1 fraction, indicating release from the cell cycle blocks.

in investigating heat shock effects on cell cycle progression. The ability to measure individual cellular amounts of DNA allowed determination of the percentage of cells in at least G1, S, and G2/M in large cell populations (cell cycle phase distribution) when using asynchronously proliferating cells. Changes in the cell cycle phase distribution indicate cell cycle blocks, phase prolongations, or accelerations (fig. 1).

Growth-stimulatory effects. In most cases, heat shock causes antiproliferative effects; however, two cases have been described in which heat shock seems to stimulate growth. In quiescent Swiss 3T3 cell, a mild heat shock 3 h prior to serum stimulation enhanced the number of cells entering S phase after epidermal growth factor stimulation without changes in the cell cycle kinetics. This positive effect is believed to arise from general inhibition of protein synthesis, which also inhibits the expression of negative growth regulators [50, 55]. Another stimulation may arise from the heat-induced expression of protooncogenes such as c-*fos* and c-*jun*. One transcription factor responsible for their induction, SRF/TCF, is activated by a mitogen- as well as a stress-inducible pathway [55, 56].

A secondary stimulatory effect has been reported for CHO cells after a 45.54 °C heat shock. The first cell cycle after the heat shock is greatly prolonged. However, the doubling time of the following cell generations up to 120 h was reduced to 61% compared to unstressed control cells [57].

Growth-inhibitory effects. Acute heat shock treatments up to 41 °C (mild heat shocks) do not cause long-lasting effects, as shown, for example, in rat histiocytoma cells [53]. However, moderate heat shocks (e.g., 45 min at 43 °C or 10 min at 45 °C) applied to asynchronous human fibroblasts, CHO, and rat C6 glioma cells induce two cell cycle blocks as revealed by flow cytometric studies. Cells become arrested in either G1 or G2 after completing cell cycle progression to these points depending on the cell cycle phase in which the cells had been exposed to heat shock (fig. 1) [57, 58; N. M. Kühl et al., unpublished data].

During the first hours of recovery, the cell cycle phase distribution does not change and the cells probably remain arrested at the position where they experienced the heat shock. This 'immediate arrest' might be due to the general heat shock effects discussed above. For example, in CHO and HeLa cells, the onset of cell cycle progression coincides with the removal of nuclear proteins that had accumulated after heat shock [59–61]. After 8–10 h of recovery, C6 glioma cells in S phase resume DNA synthesis and progress toward the G2 phase. The parallel disappearance of early S phase cells and increase in the G2/M fraction indicate that G1 and G2/M cells are still arrested in their cell cycle blocks (fig. 1, 10 h). A possible corresponding resumption of cell cycle progress in G1 or G2 and a subsequent accumulation of cells at the G1/S or G2/M checkpoint cannot be measured by DNA determination. In any case, heat shock eventually leads to a double block with nearly all cells distributed in either G1 or G2/M ('delayed arrest,' fig. 1, 16 h). After further recovery and a decline in HSP levels, cells are relieved from both blocks simultanously resulting in a quasisynchronous transition from G1 into S and from G2/M into G1. Heat shock thus synchronizes DNA synthesis and mitosis in formerly asynchronous mammalian cells consistent with the early results in *Tetrahymena* and *S. pombe* [1, 2].

Flow cytometric measurements do not distinguish G2 and M phase cells. However, microscopic investigation of C6 glioma cells clearly indicated a heat-induced block in G2 and not in M. Mitotic cells detach from the culture dish, do not bear processes, and appear bright in a phase contrast microscope. Such cells did not accumulate in heat-shocked C6 cultures during the cell cycle block. In addition, samples of these cells stained with 4′,6-diamidino-2-phenyl-indol-dihydrochloride do not show an accumuation of cells with M-phase-specific, condensed chromatin structures [N. M. Kühl et al., unpublished data].

The heat-shock-induced transient biphasic distribution of cells may suggest that S phase cells recover faster from heat shock effects than cells in the other phases. However, this is not supported either by flow cytometric studies comparing the heat-induced phase prolongation using synchronous cultures or by double-staining experiments with bromodeoxyuridine and propidium iodide. These studies reveal that all cell cycle phases are prolonged after heat shock. The extent of the cell cycle phase-specific differences depends on the applied heat shock as revealed by the results derived from CHO cells: after a 5- to 15-min heat shock of 43 °C, phase prolongation varied between 16 and 25 min per minute heat shock. Cells in G1 were most resistant, S and M phase cells showed medium sensitivity, while the largest delay was observed for cells in G2 [52]. In contrast, after a 20-min heat shock at 45.5 °C, cells remained in G1 for 28 h and in S or G2/M for 20 h. This corresponds to a sevenfold prolongation of G1 and a twofold prolongation of S and G2/M compared to control cells [62]. With these results in mind, the somewhat exceptional behavior of S phase cells may be explained by the lack of a restrictive checkpoint, such as the checkpoints in G1 and G2/M, rather than by higher resistance. Furthermore, S phase progression after a strong heat shock led to cell death in CHO and HeLa cultures, revealing this fraction to be especially sensitive to heat killing [63, 64].

Effects on differentiation. In addition to the blocks and delays mentioned above for asynchronous cultures, another antiproliferative mode of heat shock is observed when using cells synchonized by serum starvation. An acute heat shock during the first hours of serum stimulation of quiescent or G0-arrested C6 cells delays the serum-induced S phase entry for about 10 h as measured by flow cytometry [N. M. Kühl et al., unpublished data]. Microscopic examination of the morphological dedifferentiation kinetics suggests that heat shock may lead to inhibition of the G0/G1 transition. However, this transition is hard to characterize, and the heat shock effects might also be interpreted as delays in G1 progression. In agreement with a G0/G1 block, heat shock induces differentiation markers or differentiated phenotypes and enhances the effectivity of cell-type-specific inducers of differentation in HL60 cells, neuroblastoma cells, U937 leukemia cells, malignant human thyroid cancer cells, and embryonic carcinoma cells [65–70].

Lethal effects. Above a certain threshold heat shock is lethal. This lethality threshold is a function of the heat shock temperature and duration. The magnitude of the lethality threshold is cell type dependent. For example, HeLa cells are more resistant than CHO cells towards high temperature. This may be due to the high constitutive level of HSP70 expression in HeLa cells, which is reached in rodent cells only after stress [51, 54]. Even though the term 'threshold' implies an all-or-nothing reaction, heat-induced cell death is a gradual process. Depending on how much the lethality threshold is exceeded by a heat shock treatment, different proportions of cells will die in a stressed culture [53, 57]. This is well exemplified in a colony formation assay experiment determining the surviving fraction of BHK cells after different heat shocks. To obtain isosurvival after heat shocks between 43.5 and 57 °C, a 1.8-fold decrease in exposure time per degree temperature increase is required in this cell line [71].

In addition, heat shock lethality depends on two other factors: cell-cycle-phase-dependent sensitivity as well as a cell-type- or temperature-dependent mode of cell death. For a given cell line, the lethality threshold depends on the cell cycle phase at which the heat shock is applied. In general, S and M phase cells are more sensitive to heat killing than G1 and G2 cells [51, 57, 64, 72]. In CHO cells, the higher sensitivity of S phase cells is brought about by the heat-induced appearance of chromosomal aberrations, which are not seen in G1 cells [64, 73].

Even though we cannot discuss here the different modes of heat-shock-induced cell death in detail, there are some important general characteristics of cell death. Acute heat shock may induce cell death via apoptosis, necrosis, or chromosomal aberrations (loss of clonogenicity) [53, 64, 73–76]. The type of cell death seems to be cell type and temperature dependent. For example, acute heat shock of 42 or 43 °C causes apoptotic cell death in malignant rat histiocytoma cells, whereas heat shock of 45 °C induces cell death via necrosis [53]. Such a switch from apoptosis to necrosis was also observed in P815X2.1 mastocytoma and L5178Y cells [76, 77]. Furthermore, blood cells seem to undergo heat-induced cell death via apoptosis even after mild heat shock from 42 to 43 °C, whereas for glioma cells or fibroblasts, treatments up to 44 °C may not be lethal [52, 53, 74]. This may be due to the fact that in blood cells, the apoptotic pathway is also induced in response to physiological signals.

Thermotolerance development. Apart from general obstructive effects, heat shock also triggers adaptive responses. For most of the primary targets mentioned above, a mild heat shock treatment followed by a recovery phase at 37 °C protects cells from severe damage induced by a subsequent heat shock at high temperatures. This state was termed 'acquired thermotolerance' even though it can be induced by several agents and includes enhanced tolerance to other stressors, e.g., irradiation. Depending on the target, thermotolerance is observed either as protection from heat shock effects or as faster recovery after a second treatment [35, 47, 78–83].

In terms of cell proliferation, thermotolerance is generally understood as protection from cell death. This might be achieved by a shift of the lethality threshold to higher temperatures or by enhanced cell survival at the same temperature [49, 84–86]. For example, induction of thermotolerance (43 °C, 20 min) prevents chromosomal aberrations induced in nontolerant CHO cells by a 30- to 100-min heat shock treatment. Cell survival is thus greatly enhanced, and thermotolerant cells have to be exposed to a twofold dose of heat for the same amount of cell death as seen in normal CHO cells [57]. In addition to the type of thermotolerance described above, thermotolerance induction was recently shown to shorten the cell cycle arrests and prevent phase delays after acute heat shock [N. M. Kühl et al., unpublished data].

In general, thermotolerance against heat-induced cell death develops very early during the recovery from acute heat shock [49, 51, 84–86]. For example, in CHO cells, thermotolerance is detectable 4–8 h after heat shock, is maximal after about 12 h, and then decreases progressively up to 72 h [51, 57]. However, the kinetics of development and decay as well as the extent of thermotolerance depend on the particular form of the inductive heat shock.

Many studies have shown that thermotolerance develops in all cell cycle phases. Nevertheless, the extent and pace of thermotolerance development is cell cycle phase dependent [72]. G1 cells develop the highest thermotolerance, whereas M and S phase cells develop the lowest thermotolerance. Furthermore, the decay of thermotolerance is not correlated with cell cycle progression after release from the block [57, 84, 85].

Chronic (long-term) heat shock treatment

The most obvious difference between acute and chronic heat shock treatment is observed in the recovery phase: in the first case, resumption of cell cycle progression occurs after cells are returned to 37 °C, whereas cell cycle progression in the latter case occurs during the chronic heat shock treatment [51]. Cell cycle progression is then strongly influenced by thermotolerance development (adaptation) during long-term exposure (> 4 h) [51, 54, 87].

With the exception of some cell lines, mammalian cells are normally not able to proliferate during temperatures continuously elevated above 39 °C. Therefore, most investigations applying continuous exposures deal with heat-induced cell death. As in the case of acute heat shock, cell death during chronic heat shock is gradual when determined in a cell population. The surviving fraction declines as a function of heat shock duration [51, 57, 87].

In most cases, chronic heat shock induces the same cell cycle arrests in G1 and G2/M as does acute heat shock. However, the redistribution of nearly all cells into G1 or G2/M fractions arises differently as determined by flow cytometry. The loss of S phase cells is often due to heat-induced cell death instead of accumulation in G2/M. After some hours of adaptation, G1 and G2/M cells restart cell cycle progression and most die from the effects of chromosomal aberrations/loss of clonogenicity [51, 87].

In addition, a second type of redistribution has been described for cell lines that are more heat resistant and, therefore, do not arrest in response to damaging heat doses, e.g., HeLa cells. These cells show a large accumulation of S phase cells, probably due to prolongation of this phase during the first hours of heat shock. Thereafter, cells prematurely enter M phase and subsequently die [54, 82, 88].

Thermotolerance development during chronic heat shock treatment. As an extension of the definition given above, thermotolerance can be induced by acute and chronic heat shock and protects cells against a second acute or chronic heat shock. Furthermore, in approaches using chronic mild-temperature heat shock treatments, the surviving fraction develops thermotolerance during the treatment. This also protects cells from cell death induced by an immediately subsequent high-temperature heat shock [71, 87]. For example, chronic heat shock treatment at 45 °C is lethal for asynchronous CHO and HeLa cells. However, when the cells had been exposed to a 35-min heat shock at 45 °C followed by a 4- to 72-h recovery at 37 °C, cell survival during a subsequent chronic treatment was strongly enhanced [57, 84].

In contrast to acute heat shock treatments, thermotolerance development and decay are cell cycle phase dependent in studies investigating the survival of CHO cells during chronic 42 °C hyperthermic challenge [87]: thermotolerance developed faster in G1 than in G2/M cells whereas S phase cells were unable to develop thermotolerance at all. The decay of thermotolerance coincided with the entry of formerly G1-arrested cells into S phase, but cell cycle progression was not correlated with thermotolerance decay in G2/M cells.

These results as well as those from studies in yeast, in which cells in stationary phase are constitutively thermotolerant [89], may lead to the idea that thermotolerance results from cell cycle arrest. However, in view of the fact that after acute heat shock, decay of thermotolerance is not dependent on the cell cycle [84, 90; N. M. Kühl et al., unpublished data], cell cycle arrest might confer enhanced resistance in some cases but cannot be regarded as the basic mechanism of thermotolerance development. In addition, the phenomenon of acquired thermotolerance has also been attributed to the increased production of, in particular, the 70-kDa family of HSPs [35, 91–93]. In agreement with this theory, many agents which elicit stress protein synthesis also confer the thermotolerant state [92, 94] Furthermore, induction of nonfunctional stress proteins via exposure to amino acid analogs or inhibition of stress protein synthesis by cycloheximide during the recovery period prevented acquisition of thermotolerance after heat shock treatment [78, 93]. Finally, the kinetics of thermotolerance induction and decay correlated with the kinetics of stress protein synthesis and degradation [95]. The most convincing evidence, however, is represented by the fact that after transfection with *hsp70* under the control of a constitutively active or inducible promoter, overexpression of HSP70 confers thermotolerance [70, 96]. However, there are also studies that show thermotolerance induction without enhanced HSP expression [97].

Possible heat-sensitive cell cycle control mechanisms

The results of the kinetic investigations revealed, in the main, two major groups of checkpoints at the G1/S and G2/M transitions [98] where cells are ultimately arrested after heat shock. Some features of these checkpoints will be discussed below with respect to putative heat shock effects.

The first checkpoint in late G1 (also called the restriction point R) registers cell size, nutritional state, and DNA damage, probably by the activities of S-phase-initiating proteins. In higher eukaryotes, these activities depend on numerous signals. When all requirements at this checkpoint are met, DNA synthesis is initiated [99–101]. A second group of checkpoints is located at the G2/M border and within M. A first checkpoint registers the completion of DNA synthesis in G2 [102]. Another checkpoint shortly before M controls the preparatory steps for mitosis and initiates mitosis [103]. In metaphase, the association of kinetochore microtubuli with sister chromatid kinetochores is checked [103, 104]. Destruction of an inhibitor of chromosome disjunction [105] and of cyclin B represent steps necessary for anaphase A and for M/G1 transition. The checkpoints before the initiation of replication and before and after the separation of sister chromosomes during mitosis represent the most important 'points of no return,' i.e., irreversible processes within the cycle which impose a strict 'directionality' at these transitions [106].

Cdks are the 'engines that drive the events of the eukaryotic cell cycle' [108] and are essentially involved in most checkpoints. The activity of several Cdks oscillates during cell cycle progression and so they function as clocks, which time other cell cycle events. This oscillatory activity is partially due to the different cyclins that associate with Cdks and reach their highest concentrations at different phases of the cell cycle (table 2). They also determine the substrate specificity of the Cdks [108].

Cyclin D/Cdk4 and cyclin E/Cdk play decisive roles in the G1/S transition checkpoint (restriction point), which consists of several control circuits. A major control circuit acts at the transcriptional level by regulating gene expression for enzymes involved in DNA synthesis. A regulatory protein, the retinoblastoma protein (pRb and two similar isoforms), controls the activity of specific transcription factor(s) (E2F/DP) for the respective genes (fig. 2). Upon sequential hyperphosphorylation of pRb by cyclin D/Cdk4 and cyclin E/Cdk2 [109], the transcription factors are released from the complex and subsequently activate genes coding for enzymes of the replication machinery, of itself (E2F), and cyclin E [107, 109–115].

Cyclin B/Cdk1 at the G2/M transition just before mitosis undergoes a dramatic translocation into the nucleus and, once activated, drives the cell through prophase into metaphase by phosphorylating proteins that are involved in chromosome condensation, nuclear envelope breakdown, and changes in the distribution and dynamic behavior of microtubules. At the metaphase/anaphase and anaphase/telophase transitions, Cdk activity—directly or indirectly—triggers the destruction of an anaphase inhibitor and cyclin B by means of an anaphase-promoting complex [106, 107].

The Cdks are embedded in a complex regulatory network, which is able to establish a sequential order of

Table 2. Binding partners of cell-cycle-regulating Cdks in mammals [107].

Cdk	Binding to	Active period
Cdk1 (Cdc2)	cyclin A cyclin B	late S to early M early G2 to mid M
Cdk2	cyclin E cyclin A	mid G1 to mid S early S to mid G2
Cdk4 and Cdk6	cyclin D1–3	early to late G1

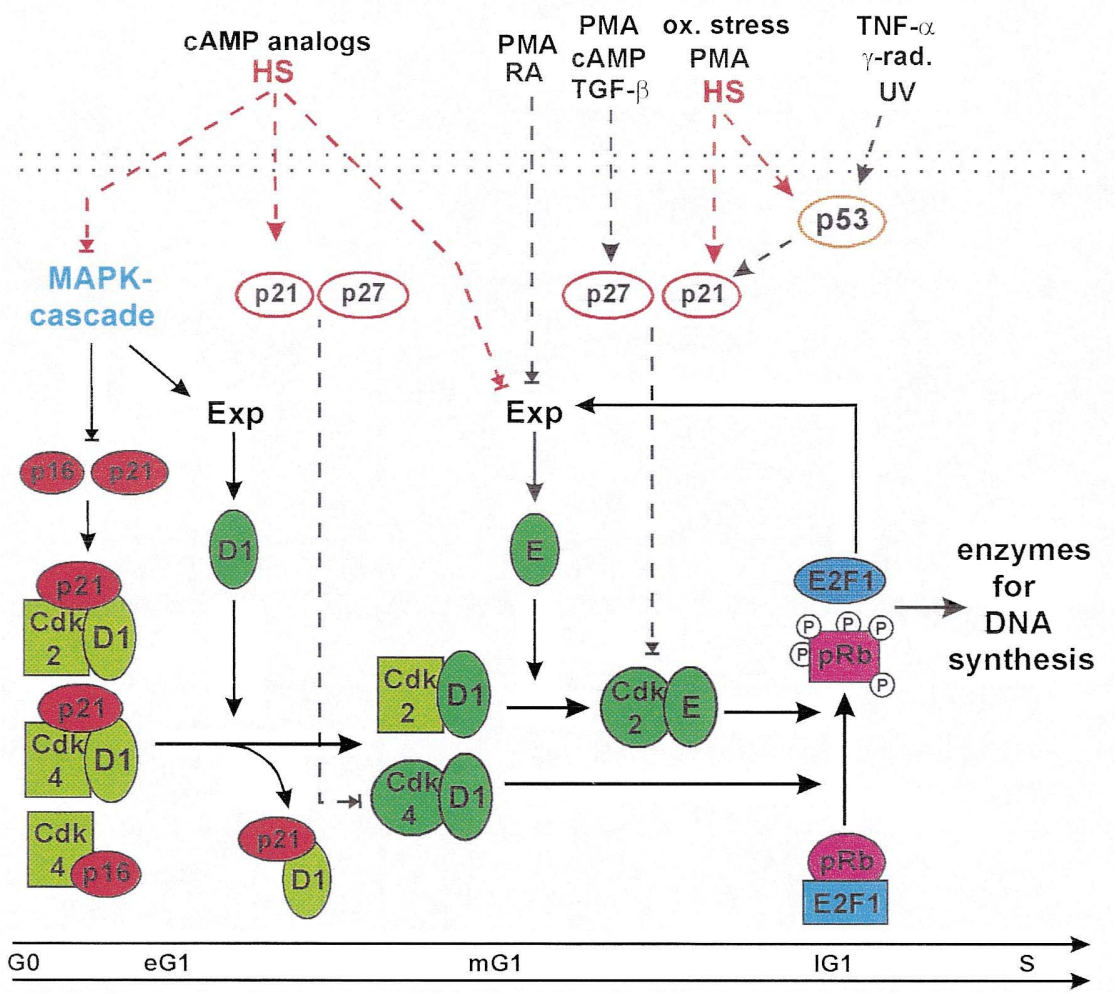

Figure 2. Putative model of the stress-induced inhibition of the G1/S transition. A major step allowing the G1/S transition is the phosphorylation of pRb mediated by Cdk4/cyclin D1 and Cdk2/cyclin E in mid to late G1. This leads to E2F1 activation which in turn activates S-phase-specific genes (for details see text). Several stressors induce a G1 arrest probably through prevention of pRb phosphorylation. In many cases the arrest is due to the induction of at least one Cdk inhibitor (p15, p16, p21, or p27). Some stressors also influence the expression of G1 cyclins. γ-rad., γ-radiation; cAMP analogs, includes elevation of intracellular cAMP; D1, cyclin D1; E, cyclin E; eG1, early G1; Exp, expression; mG1, mid G1; lG1, late G1; ox. stress, oxidative stress, e.g., hydrogen peroxide, menadione; P, phosphorylation; PMA, phorbol myristyl acetate; RA, retinoic acid. → activating or positive effect; →| inhibitory or negative effect. Arrows with dashed lines and unfilled ovals indicate stress-induced effects on cell cycle regulators. Light green, inactive; dark green, active Cdk-cyclin complexes.

their own oscillatory activity and of the downstream events such as DNA synthesis and mitosis. This regulatory network is apparently designed to react to external signals such as growth factors (transforming growth factor-β, tumor necrosis factor-α), activators of the MAPK cascade, and to perturbations by heat shock and other stressors such as UV light, γ radiation or oxidative stress (figs 2, 3). Long-term (delayed) cell cycle arrests induced by stressors may predominantly occur via regulatory mechanisms that involve thresholds. Such thresholds may reside in the amount of cyclins and the corresponding activity of Cdks or in the Cdk-dependent phosphorylation of pRb (fig. 2).

Furthermore, most Cdks are regulated by means of phosphorylation at a conserved T residue. In cyclin B/Cdk1, this phosphorylation is of particular importance. Prior to mitosis, Cdk1 is inhibited by phosphorylation of T14/Y15 in the active site. At the end of G2, Cdk1 is abruptly dephosphorylated by phosphatases of the Cdc25 family (fig. 3). During mitosis, the inhibitory kinases become increasingly inhibited whereas the phosphatase is activated by Cdk1-dependent phosphorylation. These interactions between

Cdk1 and its kinases and phosphatases form a positive feedback loop at the G2/M transition [107].

In addition to the control by synthesis and degradation of cyclins and by phosphorylation/dephosphorylation of Cdks, the activity of several cyclin/Cdk complexes is regulated by Cdk inhibitors (Ckis) [116]. In mammalian cells, two families of Ckis have been identified: the Cip/Kip and the Ink4 families. They provide regulatory mechanisms sensitive to intracellular and extracellular signals (figs 2, 3). Ckis play a crucial role as effectors of checkpoint control mechanisms and in the arrest of cell proliferation at the correct developmental stage (differentiation) and in response to DNA damage, stressors, and factors such as transforming growth factor-β [117]. They also function as tumor suppressors. Mammalian Cip/Kip inhibitors (p21, p27) bind cyclin Cdk complexes of the G1 and G1/S phases but apparently also in the G2 phase. Ink4 proteins (p15, p16) mainly inhibit Cdk4 (fig. 2) In *Saccharomyces cerevisiae*, the Cdk inhibitor Sic-1 is regulated at the level of transcription and protein degradation [118]. It is synthesized in G1 and degraded by the same S-phase-promoting complex by which G1 cyclins are destroyed. A homologous complex has also been found in mammalian cells.

Effects of heat shock on cell cycle regulators

Effects on G1 regulators. The regulatory pathways responsible for heat-induced cell cycle blocks are not yet clear, although some progress has recently been made, particularly with regard to a putative role for pRb and its phosphorylation.

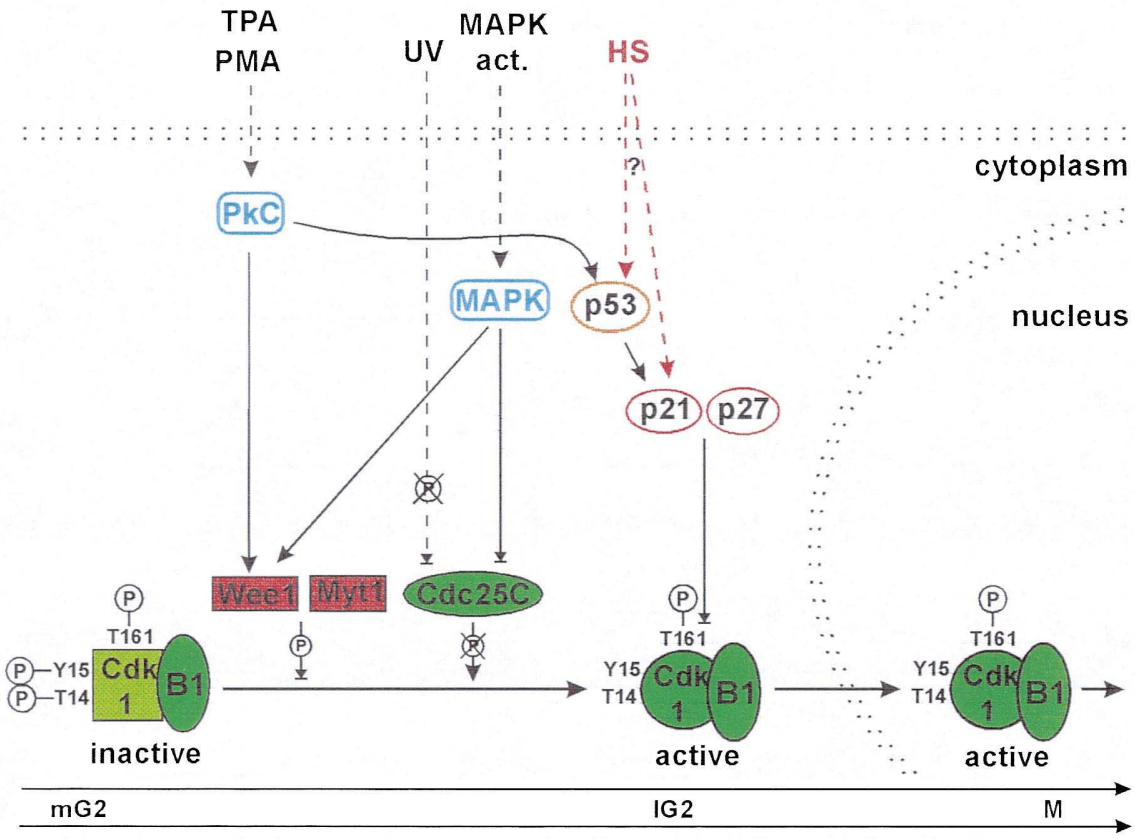

Figure 3. Putative model of the stress-induced inhibition of the G2/M transition. The major step allowing the G2/M transition is the activation and nuclear translocation of cyclin B/Cdk1. After initial activation, cyclin B/Cdk1 down-regulates its inhibitory kinases Wee1 and Myt1 and activates its activating phosphatase Cdc25C providing a positive feedback loop. Several stressors are known to interefere with Cdk1 activation thereby inducing G2 arrest. UV-induced DNA damage prevents Cdk1 dephosphorylation by Cdc25C inhibition. PkC stimulation by TPA or PMA activates the Cdk1 kinase Wee1, and MAPK activation affects both Wee1 and Cdc25C indirectly. The heat shock targets leading to G2 arrest are currently unknown. However, in analogy to the heat-induced G1 arrest, one possible pathway may be the induction of the Cdk inhibitor p21. B1, cyclin B; mG2, mid G2; lG2, late G2; M, M phase; MAPK act., activation of MAPK by transfection or microinjection of activated *raf*-1, c-*mos* or other upstream MAPK activators; P, phosphorylation; PMA, phorbol myristyl acetate; TPA, 12-*O*-tetradecanoyl phorbol-13-acetate; → activating or positive effect; →| inhibitory or negative effect. Arrows with dashed lines and unfilled ovals indicate stress-induced effects on cell cycle regulators.

Heat shock suppresses the hyperphosphorylation of pRb, thus inhibiting the release of E2F which is bound to the hypophosphorylated form of pRb. This prevents a necessary step for entry into S phase as shown in SV40-infected primary mouse kidney cells [119]. Without pRb phosphorylation, E2F-1 is not active, and the G1/S transition cannot be passed. Therefore, the loss of pRb phosphorylation may be a critical factor in G1 arrest after heat shock. Several lines of evidence indicate that upstream targets of hyperthermia are the pRb-phosphorylating Cdks (fig. 2). In human glioblastoma cells, the heat-induced G1 arrest is accompanied by an increase of p21 mRNA and protein [120]. p21 is known to bind to the cyclin-D,E-dependent kinases 4, 6 and 2. Furthermore, heat shock and several other stressors increase p53, a chief upstream regulator of p21, leading to elevated expression of p21 (fig. 2) [120–123]. Thus, one heat shock effect leading to growth arrest in G1 apparently involves inhibition of pRb phosphorylation via the p53-induced Cdk inhibitor p21. It is interesting to note, however, that p21 induction after hyperthermia also leads to cell cycle arrest in p53-deficient glioma cells [120]. These results suggest that alternative pathways may be involved in hyperthermia-induced p21 expression. One such pathway may include BRCA1, which has been shown to induce p21 in a p53-independent manner [124].

Another effect contributing to heat-induced G1 arrest seems to be based on inhibition of cyclin expression. In yeast, heat shock inhibits Cln 1 and 2 synthesis at the transcriptional levels by means of repressor Xbp1 (Xho1-site-binding protein 1) activation. Furthermore, degradation of pre-existing Cln 1 and 2 is enhanced whereas Cln 3 is unaffected [125, 126]. In synchronous rat C6 glioma cells, heat shock delays cyclin E expression by the same extent as S phase entry [N. M. Kühl, unpublished data].

A third heat shock target for G1 arrest may be the MAPK cascade. However, the results in this field are ambiguous. MAPK activation is normally attributed to growth stimulatory pathways. Heat-induced inactivation of MAPK by specific phosphatases (MKP) [127–129] is in line with the model presented in figure 2. However, in several mammalian cell lines, a short-term activation of MAPK has been reported after heat shock [130]. Lewis et al. [31], therefore, have speculated that only long-term MAPK activation, as observed after serum stimulation, acts as a positive growth signal, whereas short-term activation inhibits cell cycle progression.

Heat shock also activates the stress-regulated kinases JNK and p38 [31, 32]. However, these kinases have not been shown to contribute to heat-induced cell cycle arrest, but are thought to trigger heat-induced apoptotic cell death [31, 91].

Effects on G2 regulators. Even less is known about how heat shock leads to cell cycle arrest in G2. One possible signal pathway may be represented by the major G2/M kinase, Cdk1 (Cdc2). Poon et al. [131] found that DNA damage inhibits Cdk1 activity by T14 and Y15 phosphorylation concomitant with a reduction in Cdc25C phosphatase activity. In addition, an undefined Cdk inhibitor was reported to contribute to this cell cycle arrest [105], a possible candidate for which is p21, recently shown to act in G2 [132]. Injection of purified p21 into *Xenopus* oocytes potently inhibited the G2/M transition and the activating dephosphorylation of cyclin B1/Cdk1 [133]. Ectopic expression of p21 at physiological levels arrested cells at G1/S and G2/M in various mammalian cell lines [134]. This evidence together with the heat-induced expression of these Cdk inhibitors in G1 may suggest the inhibition of Cdk1 activation for a heat-induced G2 block (fig. 3). However, Cdk1 has not yet been identified directly as a target of the heat-shock-induced G2 arrest.

In contrast, under lethal heat shock conditions, an effect on cyclin B/Cdk1 has been demonstrated. In some cell lines, premature chromatin condensation is then accompanied by a strong induction of cyclin B expression, possibly due to severe S phase prolongation. Cyclin B induction is thought to activate Cdk1 and to induce G2 and M phase processes during S phase [88]. Besides the putative effects of p21 and p27, there may be additional inhibitory heat shock effects on Cdk1. As summarized in figure 3, artificial activation of PKC as well as MAPK in G2 may indirectly prevent Cdk1 activation. Both kinases activate pathways that lead to enhanced inhibitory phosphorylation of T14 and Y15 in Cdk1 [31, 135, 136]. Since heat shock elevates the intracellular level of free Ca^{2+} and activates MAPK at least for a short period of time, these signalling cascades may also contribute to the G2 arrest after hyperthermia [31, 62, 130]. Higher levels of HSPs may be involved in a release of C6 glioma cells from the G2/M block because such a release was observed after HSP maxima (N. M. Kühl et al. unpubl. data).

Conclusions

Heat shock treatment can probably arrest cells at any phase of the cell cycle, as concluded from the fact that the cell cycle distribution of randomly proliferating or synchronized cultures may remain constant for several hours after heat shock. When cells resume cell cycle progress after further recovery, they may be arrested again at certain checkpoints such as G1/S and G2/M, because these checkpoints may contain threshold values for Cdk activities and for the phosphorylation of their

substrate proteins such as pRb, which have to be reached before the next step is initiated. Cdk activities are determined by the amount of their respective cyclins, by Cdk phosphorylation, and by the concentration of their inhibitors. These regulatory mechanisms are affected by heat shock, for example by inhibition of the synthesis of cyclins and by an increase in the amounts of Cdk inhibitors. Cdk-dependent hyperphosphorylation of pRb and the subsequent release of E2F may provide another threshold to be overcome.

A 'priming' heat exposure renders cells more thermotolerant during a second heat treatment—either with respect to the duration of the cell cycle arrest or initiation of cell death. The same thermotolerant state which is reached during recovery from an acute heat shock seems to facilitate the resumption of cell cycle progression. Acquired thermotolerance is, at least in part, due to increased levels of HSPs. General stabilizing effects of HSPs on basic processes such as gene expression as well as more specific stabilizing functions within the cell cycle contribute to this thermotolerance. The latter is suggested by the phase-specific synthesis of HSPs, the higher level of HSPs in proliferating and transformed cells, and the complex formation between HSPs and cell cycle proteins. The exact role of HSPs within the cell cycle is, however, not yet known.

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