Use of conditioned media is critical for studies of regulation in response to rapid heat shock

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Abstract Heat shock response (HSR) maintains and restores protein homeostasis when cells are exposed to proteotoxic heat stress. Heat shock (HS) triggers a rapid and robust change in genome-wide transcription, protein synthesis, and chaperone activity; and therefore, the HSR has been widely used as a model system in these studies. The conventional method of performing instantaneous HS in the laboratory uses heated fresh media to induce HSR when added to cells. However, addition of fresh media to cells may evoke additional cellular responses and signaling pathways. Here, we compared the change in global transcription profile when HS is performed with either heated fresh media or heated conditioned media. We found that the use of heated fresh media induces transcription of hundreds of genes that HS alone does not induce, and masks or partially masks HS-mediated downregulation of thousands of genes. The fresh-media-dependent upregulated genes encode ribosomal subunit proteins involved in translation and RNA processing factors. More importantly, fresh media also induce transcription of several heat shock protein genes (*Hsps*) in a heat shock factor 1 (HSF1)-independent manner. Thus, we conclude that a conventional method of HS with heated fresh media causes changes in transcription regulation that confound the actual change caused solely by elevated temperature of cells.

Keywords Heat shock · Heat shock proteins · Transcription · Conditioned media · Gene regulation · PRO-seq · Serum · Glucose · Cell culture media

Introduction

Exposure of organisms to various kinds of cellular stresses is an inevitable consequence of living in a dynamic environment. Stresses disrupt protein homeostasis and give rise to a proteotoxic cellular environment, which is detrimental to the proper cellular functions (Morimoto 1998). To cope with stresses such as elevated temperature, heavy metals, toxins, oxidants, and bacterial and viral infections, organisms mount a rapid and robust response, known as the heat shock response (HSR). This cellular defense mechanism against stresses is evolutionarily conserved in organisms of all complexities (Lindquist 1986). The characteristic feature of the HSR is the dramatic induction of heat shock protein (HSP) genes (*Hsps*), which are molecular chaperones specialized in proper folding of de novo synthesized peptides and refolding of misfolded proteins (Morimoto 1993). Successful rescue by *Hsps* to regain protein homeostasis returns cells to their native state; however, cells subjected to a prolonged proteotoxic environment can lead to a failure of molecular chaperones to maintain proteostasis and eventually trigger cellular apoptosis pathways (Jolly and Morimoto 2000).

In mammals, the rapid and robust induction of *Hsps* upon stress is regulated by heat shock transcription factor 1 (HSF1) (Wu 1995) at the transcriptional level, which is a primary and major point of regulation (Lis and Wu 1993). Upon stress, HSF1 trimerizes and binds to the promoter of *Hsps* at three or more inverted repeats of nGAAn pentamers (Perisic et al. 1989), known as the heat shock element (HSE). HSF1 recruits co-factors that promote release of paused Pol II into productive elongation (Lis et al. 2000). Increasing evidence suggests that the number of HSF1-regulated genes exceeds the repertoire of genes referred to as *Hsps* (Trinklein et al. 2004; Vihervaara and Sistonen 2014). HSF1 and its target genes are implicated in obesity (Ma et al. 2015), longevity (Morley

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and Morimoto 2004), and cardiovascular diseases (Yan et al. 2002). More importantly, HSF1 plays opposing roles in cancer and neurological disorder (Ciocca and Calderwood 2005; Dai et al. 2007). These findings support a broad role of HSF1 and HSR in homeostasis and disease. Nonetheless, much work remains in elucidating the totality of the stress response and its interplay with disease states.

The field of stress biology has greatly benefited from using the HSR as a model to study stress response. Presence and importance of the HSR from lower eukaryotes to mammals, similarity in factors involved, and the ease of use in laboratory settings make it a highly desirable system. HSR is robust, extensive, and rapid; previous studies have documented recruitment of transcription factors and major transcriptional changes occurring within a few minutes of heat shock (HS) (O'Brien and Lis 1993; Zobeck et al. 2010). Therefore, a precise method to instantly trigger HS in laboratory settings is important to further investigate the first-order kinetic changes in HS-induced transcription regulation. To perform instantaneous HS, many labs substitute the existing cell culture media (hereafter called conditioned media) with heated fresh media for adherent cells or add heated fresh media to cells in suspension. Although this strategy achieves instantaneous HS, it also possesses a risk, as addition of heated fresh media could evoke other cellular responses beyond the HSR. Transcriptional changes induced by treating serum-starved cells with fresh media are well documented (Pirkmajer and Chibalin 2011). Similarly, nutritional lipid has potential to modify stress response in *in vitro* condition (Péter et al. 2012). As such, substituting conditioned media with heated fresh media could induce a similar response, and the potential unsolicited transcriptional changes caused by heated fresh media remain to be fully explored.

We recently reported a robust and simple method of performing instantaneous HS using heated conditioned media from the same cells growing for the same period in the same conditions (Mahat et al. 2016b), and we documented the changes in global distribution of transcriptionally engaged RNA polymerase at a base-pair resolution by our precision run-on and sequencing (PRO-seq) method (Kwak et al. 2013). In the present manuscript, we compare the use of conditioned media and fresh media in transcription profile changes during HS. We performed PRO-seq in wild-type (WT) and *Hsf1*^{-/-} mouse embryonic fibroblasts (MEFs) before and 12 and 60 min after HS with either fresh media or conditioned media, both heated to 42 °C. We found that the transcription induction of some genes is entirely dependent on fresh media but not HS when fresh media is used for HS. We also discovered that the global change in transcription profile upon HS is severely confounded by the use of fresh media. More importantly, fresh media induce transcription of heat-inducible *Hsps* in both WT and *Hsf1*^{-/-} MEFs indicating the activation of an HSF1-independent pathway for *Hsps* expression by some

components of fresh media. Our work reveals potential pitfalls in experimental design of stress response studies in laboratory settings and strongly supports the use of heated conditioned media for instantaneous HS experiments.

Material and methods

Cell culture

WT and *Hsf1*^{-/-} MEFs generated from wild-type mice and *Hsf1*^{-/-} littermate were provided by Ivor Benjamin Lab (McMillan et al. 1998). MEFs were grown in Dulbecco's Modified Eagle Medium supplemented with 10 % heat inactivated fetal bovine serum (v/v) and 1 % Penicillin Streptomycin (v/v) at 37 °C with 5 % CO₂ and 90 % humidity. Cells were grown to ~80 % confluence for HS treatment in 150 mm TC-treated and gamma-irradiated cell culture dish.

Heat shock with fresh media

To harvest non-heat-shocked cells, conditioned media from the cell plates were discarded and cells were rinsed with ice-cold PBS. To perform qPCR, cells were harvested by elution in 1 ml of Trizol. To prepare samples for PRO-seq, nuclei were isolated from the cells as described in the nuclei isolation section.

To perform instantaneous heat shock using fresh media, conditioned media from cells were discarded. Twenty milliliters of fresh media heated to 42 °C was gently added to the cell culture plate and immediately placed in a 42 °C incubator with 5 % CO₂ and 90 % humidity. The cells were heat shocked for desired time and harvested as described in the nuclei isolation section.

Heat shock with conditioned media

Non-heat-shocked cells were harvested as described above. Conditioned media from the cell plates were discarded and cells were rinsed with ice-cold PBS. To perform qPCR, cells were harvested in 1 ml of Trizol. To prepare samples for PRO-seq, nuclei were isolated from the cells as described in the nuclei isolation section.

Heat shock with conditioned media was performed as described previously (Mahat et al. 2016b). It requires two sets of identical cell plates for a HS experiment. Conditioned media from the first cell plate was collected and heated to 42 °C in water bath. The cells on this cell plate are discarded. Once the conditioned media were heated to 42 °C, conditioned media from the second cell plate were discarded and 20 ml of heated conditioned media collected from the first cell plate were gently added and immediately placed in a 42 °C incubator with 5 % CO₂ and 90 % humidity. The cells were heat shocked for desired time and harvested as described in the nuclei isolation section.

Nuclei isolation, nuclear run-on, and PRO-seq library preparation

Isolation of nuclei from WT and *Hsf1*^{-/-} MEFs, nuclear run-on reactions, and PRO-seq library preparation were performed as described previously (Mahat et al. 2016a).

Differential expression analysis

DESeq2 was used to identify differentially expressed genes (Love et al. 2014). PRO-seq reads were calculated from the body of genes (+500 from transcription start sites to -100 from polyA site) in both biological replicates, and the differential expression was calculated between NHS and HS conditions. Genes with *p* value less than 0.001 were considered to be significantly changed.

Gene ontology analysis

Differentially regulated genes in fresh media versus conditioned media were analyzed for enriched classes of molecular functions and biological processes using the database for annotation, visualization, and integrated discovery (DAVID) (Huang et al. 2009). Highly enriched and non-redundant GO terms from the PANTHER database were selected using the option provided in DAVID.

Results

Heated fresh media induce immediate early genes

Instantaneous heat shock is typically performed using heated fresh media (Fig. 1a). When working with adherent cells (such as mammalian fibroblasts and HeLa cells), conditioned media from the cells are discarded and fresh media heated to a desired temperature are added. Then the cells are placed in the heated incubator for a desired period of time (however, this practice is not always explicitly stated). When working with cells in suspension (such as *Drosophila* S2 cells and human K562 cells), adequate volume of heated fresh media is added to the existing media in order to attain the desired temperature (O'Brien and Lis 1993; Kim and Gross 2013; Zhang et al. 2014). These approaches of using heated fresh media for HS ensures the cells are instantaneously heat shocked; however, additional cellular responses and signaling pathways could be triggered by fresh serum or glucose present in fresh media and eventually confound the HSR. Therefore, to avoid the unwanted effects of fresh media for instantaneous HS, we used conditioned media for HS (Fig. 1b). For each experiment, two sets of cells are plated. Conditioned media from the first set of identically growing cells are collected and heated to a desired temperature. Once the media reach the desired temperature,

conditioned media from the second set of cells are discarded and heated conditioned media from the first set of cells are added to it, followed by incubation in a cell culture incubator set to the desired temperature for the desired time. This approach ensures instantaneous HS and eliminates the possibility of unwanted transcriptional changes that could be caused by the use of fresh media.

To examine the effect of fresh media, we measured transcription change on a set of immediate early (IE) genes (Esnault et al. 2014). Addition of fresh media after serum-starvation immediately and transiently induces transcription of these genes (Schratt et al. 2001). To distinguish the contribution of fresh media from HS, we treated MEFs in three different ways, (i) adding fresh media without HS, (ii) adding fresh media and HS, and (iii) adding conditioned media and HS, and then examined the changes in a transcription level of eight IE genes compared to NHS cells (Fig. 1c). We found that the transcription induction of these genes is dependent on fresh media, and HS has little to no effect on their transcription induction. This observation highlights the confounding effects caused by the use of fresh media for instantaneous HS in transcriptional induction of at least a few genes.

Fresh media causes significant change in genome-wide transcription profile

To examine the genome-wide transcriptional changes caused by the use of heated fresh media for HS, we performed PRO-seq using heated fresh media and compared with PRO-seq that was performed using heated conditioned media (Mahat et al. 2016b). We found that the fresh media used in HS strongly induce transcription of some IE genes, confirming the qPCR results. For example, transcription induction of *Nr4a1* gene is entirely dependent on fresh media, whereas transcription of *Vcl* gene is slightly induced by HS alone, which is further increased by fresh media (Fig. 2a).

We measured the PRO-seq density change in all genes prior and after HS using DESeq2 (Love et al. 2014). An additional number of genes are upregulated at both 12 and 60 min of HS when heated fresh media are used instead of heated conditioned media (Fig. 2b). Moreover, ~25 % of actively transcribing genes are downregulated after 12 min of HS using fresh media; however, many of these genes are no longer downregulated at 60 min of HS indicating the transient nature of downregulation caused by the fresh media. In contrast, transcription downregulation during HS using conditioned media is gradual, and by 60 min of HS, ~55 % of the actively transcribing genes are repressed.

To identify genes that are dramatically affected by the condition of media used for HS, we analyzed significantly upregulated genes at 12 min of HS and significantly downregulated genes at 60 min of HS in either fresh or conditioned media (Fig. 2c). This side-by-side visualization of regulated genes in

Fig. 1 Fresh media transcriptionally induce IE genes. **a** Schematics of the conventional method of instantaneous HS.

Conditioned media are discarded from cells and fresh media heated to the desired temperature are added to instantaneously HS the cells. **b** Schematic of HS method using heated conditioned media. Conditioned media from one of a pair of identically growing cell cultures are collected and heated to the desired temperature. Conditioned media from the second set of cells are discarded and heated conditioned media are added to instantaneously HS the cells. **c** Relative change in transcription of eight IE genes as measured by RT-qPCR in three conditions: (i) fresh media for 12 min at 37 °C, (ii) fresh media for 12 min at 42 °C, and (iii) conditioned media for 12 min at 42 °C. Error bars represent standard deviation from three qPCR replicates

two different media conditions allows characterization of genes that undergo complete reversal in regulation. While some genes upregulated at 12 min of HS in conditioned media are also upregulated in fresh media, many genes downregulated in conditioned media are upregulated in fresh media (Fig. 2c, left panel—blue bar), and a few genes upregulated in conditioned media are downregulated in fresh media (Fig. 2c, left panel—magenta bar). Similarly, some genes that are significantly downregulated at 60 min of HS in conditioned media are upregulated in fresh media (Fig. 2c, right panel—purple bar). These three classes, marked by magenta, blue, and purple bar, represent genes whose activities are either induced or repressed depending on the external cues.

Intrigued by this dramatic change in transcriptional regulation that is dependent on the media used for HS, we examined the above three classes of differentially regulated genes for their cellular functions using gene ontology (GO) analysis. The upregulated genes only in fresh media at 12 min of HS (blue bar in Fig. 2c) are enriched for ribosomal subunits, its structural components, and translation (Fig. 2d). In contrast, upregulated genes only in conditioned media at 12 min of HS (magenta bar in Fig. 2c) are enriched for zinc ion binding proteins, negative regulators of transcription, and focal adhesion genes.

Similarly, downregulated genes in conditioned media at 60 min of HS but induced in fresh media (purple bars in Fig. 2c) are enriched for RNA binding, RNA processing, and ribosomal structures involved in translation machinery (Fig. 2e). While these findings conform the general consensus in the field of stress biology that global transcription and translation are repressed during HS (Anckar and Sistonen 2011), it also indicates that the fresh media override the HS-mediated repression and in turn activate these genes involved in increase messenger RNA (mRNA) and protein production. Overall, this observation further supports our hypothesis that heated conditioned media should be used for instantaneous HS to avoid the confounding regulation caused by fresh media.

Fresh media induces a subset of *Hsps* in an HSF1-independent manner

The broad class of *Hsps* includes 75 genes that play a role in protein homeostasis (Kampinga et al. 2009). To dissect the role of fresh media in transcription regulation of *Hsps*, we examined the PRO-seq density change in all 75 genes upon HS with heated fresh media or heated conditioned media. In order to access the role of HSF1 in transcription regulation of *Hsps*,

Fig. 2 Genome-wide transcription profile change caused by fresh media is different than caused by HS. **a** Genome-browser screenshots of PRO-seq density in three conditions with two biological replicates each. PRO-seq density in sense and antisense directions is shown in blue and red, respectively. Small vertical black bars at the bottom represent the genomic regions that do not map uniquely at 36 bp resolution. **b** Number of significantly changed genes using heated fresh media or heated conditioned media for HS. Upregulated genes are shown in green and downregulated genes are shown in red. **c** Heatmap showing the status of PRO-seq density change in all genes that show significant upregulation at 12 min of HS with either fresh or conditioned media (left) and all genes that show significant downregulation at 60 min of HS with

either fresh or conditioned media (right). The color bars represent genes that were examined in gene ontology analysis in **d**. **d** Gene ontology analysis of enriched molecular functions and biological processes in significantly upregulated genes at 12 min of HS with fresh media but significantly downregulated in conditioned media is indicated by the blue bar, while significantly downregulated genes at 12 min of HS with fresh media but significantly upregulated in conditioned media are indicated by the magenta bar. **e** Gene ontology analysis of enriched molecular functions and biological processes in significantly upregulated genes at 60 min of HS with fresh media but significantly downregulated in conditioned media (purple bar)

we also performed PRO-seq in Hsf1 knockout (*Hsf1*^{-/-}) MEFs. We focused our analysis on 48 genes that are induced in at least one time point or cell type during HS. Approximately thirty-three percent of these genes, including major *Hsps*, are robustly induced in WT MEFs while almost all of the 48 genes are either downregulated or unchanged in *Hsf1*^{-/-} MEFs in conditioned media (Fig. 3a). In contrast, most of these 48 genes are transcriptionally induced during HS with heated fresh media (Fig. 3b). More importantly, transcription of these genes is also induced in *Hsf1*^{-/-} MEFs. The profile of HSF1 binding to the promoter of the genes (Fig. 3c) and the presence of HSE in the corresponding promoters (Fig. 3d) resembled the induced *Hsps* with conditioned media, which further indicate that the induction of *Hsps* with fresh media is mediated by factors other than

HSF1. Together, these findings reveal the potential of some components of fresh media to transcriptionally induce *Hsps* in HSF1-independent manner that are either repressed or unchanged during a proper, conditioned-media HS.

Discussion

HS is widely used as the model system to study stress response. It triggers a rapid and robust transcriptional response, and it is convenient to use in a laboratory setting. However, the widespread approach of using heated fresh media for instantaneous HS carries a risk of undesired transcriptional changes. Recently, we demonstrated a simple but effective

Fig. 3 Fresh media induce many *Hsps* in HSF1-independent manner. **a** PRO-seq density change during HS with conditioned media in 48 genes within the broad HSP family in WT and *Hsf1*^{-/-} MEFs. **b** PRO-seq density change during HS with fresh media in the corresponding 48 genes in WT and *Hsf1*^{-/-} MEFs. **c** HSF1 fold enrichment, as measured by HSF1 ChIP-seq, in the promoter of the corresponding 48 genes in WT MEFs. **d** HSE motif match score in the promoters of the corresponding 48 genes

method of performing instantaneous heat shock using conditioned media from same cells growing in identical conditions (Mahat et al. 2016b). Here, we now report the changes in transcription profile during HS when heated fresh media or heated conditioned media are used for instantaneous HS. In genome-wide transcriptional analysis using PRO-seq, we found that fresh media cause HS-independent transcription induction of additional genes and also mediate HS-independent transient repression of several genes. These fresh-media-dependent genes are enriched in ribosomal machinery components and mRNA translation. More importantly, we found that fresh media induce many *Hsps* that are either repressed or unchanged transcriptionally during HS and this induction potential of fresh media is independent of HSF1. We also discovered that the heated fresh media used for instantaneous HS strongly induce some IE genes in addition to *Hsps*.

One defining feature of IE genes is the presence of serum response element (SRE) in their promoters, and all eight IE genes examined in this study contains strong SRE. In serum-stimulation studies, serum response factor (SRF) binds to SRE and transiently induces expression of IE genes. Therefore, it is possible that either fresh serum or fresh glucose in fresh media activates SRF, which is responsible for the dramatic induction of IE genes in this study. Nevertheless, some of the eight IE genes also showed small but detectable induction by HS alone. Because the common feature of these genes is the presence of SRE in their promoters, the HS-dependent mild induction of these genes is also likely mediated by SRF. In fact, we recently identified SRF as a novel transcription factor activated by HS, which transiently binds to the promoters of transiently induced genes during HS (Mahat et al. 2016b). Based on these findings, we believe that HS itself is also capable of activating SRF. However, the mechanism of SRF activation by fresh media and HS could be different. Addition of fresh media after serum-starvation activates mitogen-activated protein kinase (MAPK) pathway and SRF is a known target of MAPK pathway (Heidenreich et al. 1999). Whereas, the interaction of SRF with the nuclear imported MAL, which is released from monomeric actin (Miralles et al. 2003), possibly due to actin polymerization during HS, is the proposed mechanism of SRF activation during HS (Mahat et al. 2016b).

Fresh media transcriptionally induce another class of genes, which encode components of ribosomal machinery involved in mRNA translation. This could be an indication of likely increased demand in translation caused by the activation of cell proliferation signaling pathways by fresh media. Fresh media also induce some *Hsps* in an HSF1-independent manner, whereas HS only induces the major *Hsps*. One likely candidate for the broad induction of *Hsps* by fresh media is the activator protein 1 (AP-1) transcription factors (TFs). AP-1 TFs are activated by serum stimulation (Poimenidi et al. 2009), and AP-1 binding element is highly enriched in the proximity of HSEs present in the promoters of *Hsps* (Mahat et al. 2016b). The highly

significant enrichment of AP-1 motifs around HSEs suggests either collaboration or redundancy between AP-1 and HSF1 in induction of *Hsps*. When fresh media is added, perhaps cells trigger HSF1-independent induction of *Hsps* through AP-1 in order to chaperone the influx of newly synthesized proteins to support cell proliferation.

The unwanted transcriptional changes caused by fresh media after instantaneous HS have eluded detection until now for several reasons. First, the major HSF1-dependent *Hsps*, which are the primary focus of HS studies, are induced to a similar level when either fresh media or conditioned media are used for HS. Second, genome-wide assays such as RNA-seq and mass-spectrometry have contributions from post-transcriptional regulatory steps of mRNA stability and translation efficiency respectively and may not reflect the precise change in the transcription level. Third, genomic tools with sensitivity required to detect small changes in nascent transcription were unavailable until now. In the last few years, highly sensitive genomic methods to quantify nascent transcription have been developed. These methods, such as GRO-seq (Core et al. 2008), PRO-seq (Kwak et al. 2013), and NET-seq (Churchman and Weissman 2011), will help to investigate the outstanding questions in the field of stress response. Because of the sensitivity of these assays, careful consideration should be taken in experimental setups, and at the very least, use of heated fresh media for instantaneous HS should be avoided.

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