Introduction

Heat shock proteins (HSPs) are molecular chaperones. They protect cells from extreme physiological, pathological and environmental conditions [1]. The heat shock response was first discovered in 1962 as chromosomal puffs in heat-shocked drosophila salivary gland cells [2]. For over 40 years, research has been pursued to understand HSP molecular structure, function, gene regulation and protein misfolding correction mechanism. In an unstressed cell the constitutively expressed HSPs regulate protein folding, protein translocation across membranes, assembly and disassembly of protein oligomers, degradation of aged proteins, etc. Furthermore, inducible HSPs can correct protein misfolding and preserve immature polypeptides from aggregation under stresses. An increased flux of non-native proteins under the stress conditions triggers the heat shock response, which results in the rapid synthesis of heat shock proteins. The elevated HSPs repair protein damage and facilitate normal cell growth conditions.

The cytoprotective functions of HSPs have been found in many organs: heart [3], brain [4], kidney [5], intestine [6,7], embryo [8], etc. Altered HSP expression has been associated with protection in many stress conditions, such as ischemia and reperfusion damage, cardiac hypertrophy, fever, inflammation, metabolic diseases, infection, cell and tissue trauma, aging, and cancer [9]. Transient exposure to elevated temperature or other selected stress sources leads to a protective effect via increased HSP expression against long-term exposure to lethal stresses.

Among the various HSPs, HSP70, with a molecular weight of 70 kDa, is known to be the major molecular chaperone in all the cellular compartments and organs. Much effort has been directed to evaluate the importance of HSP70 for long-term ischemia survival in hearts and brains using external stress preconditioning, such as heat shock, to induce HSP70 expression. For example, both whole-body hyperthermia [10] and local heating [11] of a heart increase the myocardial HSP70 level and protect against ischemia-reperfusion injury in cardiac surgery.

The accepted safe ischemic time during cardiac surgery has remained at about four hours over the past 30 years, though techniques, such as cardioplegia and hypothermia, have been developed in an attempt to prolong it. The same ischemic time is a major limit in the practice of cardiac surgery and cardiac transplantation. It has been found that the ischemic infarct size can be reduced in direct proportion to the amount of HSP expressed in the heart tissue [12]. This result suggests that enhancing the amount of HSP70 expression is an effective approach to increase the level of myocardial protection. It is important to control the thermal stress protocol during heat shock induction to be able to cause a high level of HSP70 expression. Therefore, a full understanding of the temperature and time-driven kinetics of HSP70 expression is necessary to design a propitious protocol for inducing protection. There exist some brief reports on the time kinetics of HSP70 expression [3,4]. However, comprehensive HSP70 expression kinetics data of heart tissue or cardiac related cells is not available currently.

Bovine aortic endothelial cells (BAEC) were chosen as the subject for the current study to represent the stress behavior in cardiac tissue. In an alternate model study in our laboratory, preliminary experimental data for chicken embryo myocytes demonstrated very little HSP70 increase after heat shock (unpublished), as verified by other researchers [13].

Materials and Methods

Cell Culture. BAECs (GM07372A) from Coriell Cell Repositories (Camden, NJ) were cultured with Eagle’s MEM (Sigma, M-0643) with 10% FBS, 1X MEM vitamins (Gibco, 11120-052) and 1X penicillin-streptomycin (Gibco, 15140-122). Cells were grown in a 5% CO2 incubator in 10 cm culture dishes until they were 90% confluent. Before heating, the medium was changed.

Heat Shock Experiments. Cells culture dishes were transferred for heating into a 42°C incubator and subsequently returned for recovery in a 37°C incubator, both with 5% CO2. The heating time was measured from when cells were transferred into the 42°C incubator to the time they were removed. A matrix of heating and recovery times was used: heating from 0.5, 1, 1.5, 2, 3, 4, 5 hours and recovery from 0 to 48 hours (0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 16, 24, 48 hours). After heating and recovery cells were lysed immediately in lysis buffer that has 10% SDS and protease inhibitors, and supernatant solutions were stored in a −20°C freezer for later analysis. Each set of experimental conditions for the entire heating and recovery matrix was repeated five times.

In order to investigate influence of temperature on HSP70 expression, a separate set of experiments were conducted for cell cultures in replicates of four heated at temperatures from 40°C to 44°C for two hours followed by a uniform recovery period of 14 hours. In an additional protocol, cell cultures were heated continuously for times from 0 to 48 hours at 42°C in replicates of four, followed by immediate HSP70 analysis.

HSP70 Expression Analysis. Western blot analysis was used in all samples to determine the expression of inducible HSP70. Total protein concentration was measured with Protein Assay (Bio-Rad, 500-0002) and a spectrophotometer at 595 nm (Beckman, DU 530). An equal amount of total protein was loaded into each well for electrophoresis, and the separated proteins were

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transferred from gels to PVDF (polyvinylidene difluoride) membranes by blot-transfer. A control sample, which was not heated, was included in each experiment and loaded in each gel. Ready Gels (Bio-Rad, 161-1101) and Criterion Precast Gels (Bio-Rad, 345-0009) were used in electrophoresis. After blocking in 3% nonfat dry milk blot buffer for one hour at room temperature to remove nonspecific proteins, membranes were incubated with primary antibodies: anti-HSP70 and anti-actin (Santa Cruz, sc-1060 and sc-1616) for one hour at room temperature, followed by one-hour incubation with anti-goat IgG-HRP (Santa Cruz, sc-2020) at room temperature. Proteins were visualized with chromogenic substrate, TMB (tetramethylbenzidine, Vector Laboratories, SK-4400). Membranes were dried in air and scanned digitally. Protein bands were analyzed quantitatively using Image-Pro Plus (Media Cybernetics). The linearity between the TMB intensity and HSP70 concentration was verified by a separate western blot analysis with a series of known concentrations of pure HSP70 (Stressgen, SPP-758). Protein amount was represented by the area under the intensity histogram of the protein band. The HSP70 concentration was represented by the normalized ratio as the value of measured HSP70 to actin to equalize the total protein loading for each sample. The final data exported from Image-Pro Plus was analyzed in Microsoft Excel. Western blot in duplicate was performed for all samples.

Estimation of Effective Heating Time. Cells were heated in a water-jacketed cell culture incubator. Due to slowness of the natural convection heating mechanism, cell cultures experienced an extended time constant to reach the designated temperature after sample transfer. In order to calibrate for an effective heating time defined by a step change to 42°C, an Arrhenius integral was used to represent the HSP70 expression up to two hours heating at 42°C followed by 12 hours recovery at 37°C as a function of the temperature and time:

$$K_{HSP70}(\tau) = \begin{cases} 1 & \text{when } T \leq 37°C \\ \int_0^\tau e^{-\Delta E RT(t)/a}dt & \text{when } T > 37°C \end{cases}$$

where $K_{HSP70}$ represents the HSP70 expression normalized to an unheated control, $A$ is a scaling factor (s⁻¹), $\tau$ is the total heating time (s), $\Delta E$ is an activation energy representative of the expression process kinetics (J/mole), $R$ is the universal gas constant (8.32 J/mole K), and $T$ is the temperature of the cell culture (K), which is a function of time.

$T(t)$ was measured in a 10 cm dish with water in the incubator with a thermocouple (Omega, HH64) until the temperature reached 42°C. The measured water temperature data and its fitted curve: $T(t) = 0.8755^*Ln(t) + 307.5$, are shown in Fig. 1. $A$ and $\Delta E$ values were extracted by a Matlab program using its built-in function, interp, to fit Eq. (1) to the experimental data. The integral in Eq. (1) was calculated for the measured temperature history, and an effective heating time ($t_{eff}$) was then determined for a step change to 42°C that would produce the same integral.

$$t_{eff} = \frac{K_{HSP70} - 1}{A*e^{-\Delta E/R(T(42)+273)}}$$

t$_{eff}$ is always smaller than the corresponding actual incubator heating time ($t_{act}$). In Fig. 2, the correction factor is presented as the ratio of $t_{eff}$ to $t_{act}$. For heating times, the correction factor approaches the value 1.0 as the initial transients are a smaller fraction of the total process. For actual incubator heating times of 0.5, 1, 1.5, 2, 3 and 4 hours, the correction factors are 0.48, 0.63, 0.73, 0.82, 0.95 and 1 correspondingly.

Results

HSP70 Expression Kinetics: Time Effects. Figure 3 shows western blot results of HSP70 expression for either a variable heating time with fixed post-heating period, or fixed heating time with variable post-heating period. A pure 200 ng or 150 ng HSP70 sample was added to each gel as a control for protein analysis. Fig. 4 shows the levels of HSP70 concentration for the complete matrix of heating (42°C) and post-heating (37°C) times measured as a 3-D contour map generated in Matlab using its built-in function, surf, from discontinuous experimental data points. The built-in function, interp, was applied for color shading effects within a surface patch based on a bilinear (2-D) interpolation of local data values. Each data value was an average from five independent experiments. The results demonstrate clearly that the HSP70 expression is a function of both heating time (thermal stress) and post-heating time (post-stress). The maximum HSP70 expression is about ten times the basal level and occurs at about 1.6 hours effective heating at 42°C followed by approximately 12 hours post-heating at 37°C.

Two serial concentration peaks typical of HSP70 expression kinetics can be seen explicitly in Fig. 5. Thermal stress triggers...
HSP70 gene transcription and translation. After the thermal stress is completed, HSP70 expression increases to the first peak at about five hours post-stress, drops to a minimum at eight hours, and increases again to the second peak at about 12 hours. The second peak sustains at high level for several hours followed by a slow drop. At 24 hours post-stress, HSP70 concentration is still about eight times greater than the basal level. Statistical analysis shows that the HSP70 expression at eight hours recovery was significantly lower than at 16 hours ($p < 0.05$), and the confidence level was 80% that it was significantly lower than at 5 hours ($0.1 < p < 0.2$).

The same pattern of bimodal HSP70 expression kinetics is shown in Fig. 6, which depicts the HSP70 expression for continuous heating at 42°C for times to 48 hours. The HSP70 expression at eight hours heating was significantly lower than the first peak at three hours heating ($p < 0.05$) and the second peak at 14 hours heating ($p < 0.05$). Therefore, the data for both heating followed by recovery and continuous heating confirm the existence of bimodal expression kinetics. The variability in data is somewhat large in Fig. 5 than in Fig. 6, which is possibly a result of using multiple cell culture batches for the experiments reported in Fig. 5 and a single batch in Fig. 6.

HSP70 Expression Kinetics: Temperature Effects. Figure 7 shows different levels of HSP70 expression for heating for a fixed time (two hours) at temperatures from 40°C to 44°C. At 40°C or 41°C, the HSP70 expression level was about five times greater than the control ($p < 0.01$), but there was no significant difference between the two. At 42°C, the HSP70 expression is more than eight times larger than control, and is 10 times at 43°C. At 44°C, cells became rounded and detached from the culture dish, indicating necrosis, and no HSP expression was measured.

**Discussion and Conclusions**

The kinetics of thermally induced HSP70 expression occur as a function of thermal stress temperature and time as well as post-stress recovery time, which is clearly an important component of the process. The optimal time range to achieve a 10-fold increase of HSP70 expression in BAECs at 42°C is about 90-min heating followed by 12- to 16-h post-heating. Even after 24 hours recovery, the concentration is still about 8 times higher than control. The shape of the HSP70 expression kinetics curve exhibits a two-peak phenomenon, which is consistent with the “second-window protection phase” for ischemic preconditioning observed by oth-
ers [14,15] and unveils at least one rationale for the existence of a biphasic protection effect of ischemic and heat stress pretreatment [16,17].

This data for HSP70 expression kinetics address an unresolved issue of whether absolute level of HSP70 is directly correlated with the degree of myocardial protection. Hutter [18] and Donnelly [12] showed a direct relation between HSP70 concentration and protection at 24 h recovery after stress at different temperatures (40°C to 42°C) or different levels of mild ischemic preconditioning. However, Cornelussen and Snoekx [17] concluded from their data that HSP70 concentration was not uniquely correlated with improved ischemia tolerance of the treated hearts. They found almost the same HSP70 content in the heart after both six- and 24-h recovery with the same doses of stress, but increased protection at 24 hours. According to the time kinetics curve of HSP70 expression in this paper, one possible explanation is that the high HSP70 expression level at six hours recovery is not stable, and only the second peak provides the sustained expression necessary for effective cardioprotection. Therefore, it may be that in addition to the absolute level of HSP70 induction, the duration of a high HSP70 concentration is also important for tissue protection. Further experiments will be needed to identify the underlying molecular mechanism of this two-peak phenomenon of the HSP70 expression.

The significantly higher HSP70 expression induced at 42°C and 43°C may indicate a threshold of thermal dose for maximum HSP70 expression. However, the heat shock protocol identified in this study of 90 min at 42°C is not clinically practical. Heating methods at higher input power that can produce thermal stress in a shorter time are more desirable. Therefore, heating methods with significantly higher thermal input rates, such as by laser, ultrasound and microwave sources, might be applied to identify whether there exists an influence of heating rate on HSP70 expression. Quantitative analysis of the correlation between the absolute amount of HSP70 expression and the levels of myocardial protection after a long recovery time, such as 16 to 24 hours, will be required to identify a clinically applicable heating source.

Incubator heating as used in this study has advantages and disadvantages for evaluating HSP70 expression kinetics. The incubator provides a very stable and controlled in vitro environment for cell cultures, in which temperature is the only variable. For example, CO2 partial pressure is maintained constant. Since HSP70 can be expressed in response to many kinds of stress, it is important to be able to control thermal effects from other sources of stress. The primary disadvantage of incubator heating is its long time constant to reach the desired temperature after the specimen is positioned within. Also, using western blot to assess HSP70 concentration means that every measurement is the end of an experiment, and it is not possible to observe HSP70 expression dynamically. We are implementing experiments on a light microscope with a perfusion heating stage and a transacted cell line containing a fluorescent indicator tagged HSP70 gene to reduce the heating ramp time by more than an order of magnitude and to follow the expression process continuously in real time. This system also enables localization of HSP70 expression within the cytoplasm.

In summary, our results provide a comprehensive data set of kinetics for endogenous HSP70 expression. More work is needed before the cytoprotection function of HSP70 can be effectively utilized in clinical applications.

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References


Fig. 7 HSP70 expression at 40–44°C heat shock for two hours followed by 14 hours 37°C recovery, each column bar is presented by its average±SEM, n=4.