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Inducible and constitutive HSP70s confer synergistic resistance against metabolic challenges ☆

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ABSTRACT

Chaperonic proteins, including inducible HSP70 (HSP70i) and constitutive HSP70 (HSC70), have been implicated as essential players in the cellular adaptive protection. Ensuing studies demonstrated that overexpression of either protein individually protects against thermal and oxidative challenges. The present study aimed to determine whether a concurrent overexpression of both HSC70 and HSP70i confers a better metabolic protection than the expression of each protein alone. Using a rat heart-derived H9c2 cardiac myoblast cell line, we found that HSP70i was rapidly induced within 2–8 h following a mild thermal preconditioning (43 °C for 20 min) in both parental cells and an established H9/70c clonal sub-line overexpressing HSC70. The level of HSP70i protein in heat pretreated H9/70c clonal cells reached only 50% of that in heat pretreated H9c2 parental cells. Nevertheless, protection against lethal hyperthermia, menadione (an oxidant) and hydrogen peroxide (H_2O_2) exposure in the pretreated H9/70c clonal cells was significantly higher than the sum of protection afforded by the early induction of HSP70i in the pretreated parental cells and protection afforded by the pre-existing HSC70 in the H9/70c cells without preconditioning. Using dosimetric analysis, we also found that menadione resistance in the pretreated parental cells increased linearly with cellular HSP70i level (10–300 ng/mg total protein). However, the resistance in the pretreated H9/70c cells showed a biphasic relationship with cellular HSP70i level; when HSP70i concentration reached >250 ng/mg protein, survivability after menadione exposure was markedly enhanced. Similar results were observed in H9c2 cells genetically manipulated to overexpress both HSC70 and HSP70i. The survival benefit against lethal hyperthermia, oxidant treatment, and hypoxia/reoxygenation conferred by a concerted HSC70 and HSP70i overexpression was greater than the sum of benefits contributed by individual protein overexpression. Together, these findings suggest that HSC70 and HSP70i may complement each other in a synergistic manner to preserve cellular integrity during metabolic challenges.

1. Introduction

The constitutive 70-kD heat shock protein (HSC70, also abbreviated as hsc70, hsp70c or hsp73) and the inducible 70-KD heat shock protein (HSP70i, also noted as hsp70i, hsp70 or hsp72) are two prominent members of the HSP70 chaperone family. These two proteins are highly conserved in amino acid sequences, but each manifests a distinct pattern of expression. So far, their roles in cellular defense mechanisms are still inconclusive. Using H9c2 cardiac myoblasts as a simplified myocardial model, Su and colleagues previously have shown that cells pre-heated at 43 °C for 20 min developed a resistance to subsequent H_2O_2 exposure [14]. This resistance is characterized by an early onset of prolonged protection against exposure to a low concentration of H_2O_2 followed with a development of transient protection against exposure to a moderate concentration of H_2O_2. The protection against mild H_2O_2 toxicity is associated with the immediate induction of HSP70i after thermal pretreatment, while the protection against moderate H_2O_2 toxicity is detectable within a time frame in which both endogenous HSC70 and HSP70i concentrations were augmented. Oxidative treatments are known to affect membrane integrity, denature proteins and catalyze lipid peroxidation. Therefore, our findings that HSP70s can ameliorate the injurious effects of H_2O_2 are in line with reports showing that a timely overexpression of

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both HSP70i and HSC70 in the heat-preconditioned myocardium is coincident with an adaptive protection against ischemia/reperfusion injury [7,10].

Based upon these observations, we hypothesize that HSC70 and HSP70i act collaborately to provide an optimal preservation of cellular functions during metabolic stress. A research strategy to test this hypothesis is to "increase" the levels of these two proteins simultaneously in the target cells and then assess resultant changes in these cells tolerance to environmental insults. We thermally pretreated the already established HSC70-overexpressing clonal cells or genetically inserted into the parent cells with both HSP70i and HSC70 cDNAs. The results showed that a concurrent up-regulation of these two chaperonic proteins by either approach bestowed on the target cells a remarkable resistance against lethal hyperthermia and oxidative challenges. Furthermore, the resistance in HSC70/HSP70i double transfectants was significantly greater than a simple summation of the resistance found in single transfectants. Our data support the tested hypothesis that HSC70 and HSP70i collaboratively augment cellular defense against oxidative insults.

2. Materials and methods

2.1. Cell culture

HSC70-transfected H9c2 cardiac myoblast (H9/70c-1) cells and the vector-transfected control (H9/sham cells) were established as described previously [6]. These cells were maintained in Dulbecco's modified Eagle's (DME) medium (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum, sodium bicarbonate and antibiotics under 95% air and 5% CO_2. The H9/70c-1 cells were maintained in the continuous presence of 300 μg/ml of the neomycin analogue, G418. Only exponentially growing cells were used in subsequent experiments. Two days prior to thermal pretreatment, cells were seeded at a density of $3–5 \times 10^3$ cells/cm^2.

2.2. Plasmid construction and transfection

A cDNA encoding the human HSP70i protein, pH2.3 [9], was obtained from Dr. R. Morimoto (Northwestern University, Chicago, IL). The 2.4-kb fragment containing the entire coding region was released from pH 2.3 by BamHI and HindIII double digestion and was inserted between the BamHI and HindIII sites in the pcDNA3.1/Hygro polylinker region (Life Technologies, Carlsbad, CA). In the resulting construct, designated as pHyg-HSP70i, transcription of the HSP70i cDNA was regulated by a human cytomegaloviral enhancer-promoter. Subsequently, the expression plasmid pHyg-HSP70i, or the control plasmid pcDNA3.1/Hygro was transfected into either parental H9c2 cells or the previously established H9/70c-1 clonal cells using Lipofectamine 2000 (Life Technologies, Carlsbad, CA). Approximately 5×10^5 H9/70c-1 or H9c2 cells were incubated with Lipofectamine 2000 mixed with 7.5 μg of the plasmid of interest for 12 h. After incubation, the cells were replated at a density of $5–10 \times 10^4$ cells per 100×20-mm culture dish. Stable double-transfectants harboring both pHyg-HSP70i and pCMV-HSC70 were selected with both 200 μg/ml of hygromycin B and 300 μg/ml of G418, for 2 weeks. Stable transfectants retaining the control plasmid, pcDNA3.1/Hygro, were selected with 200 μg/ml of hygromycin B for 2 weeks. Transfected cells were then singly cloned and propagated in culture medium supplemented with 300 μg/ml of G418 and/or 100 μg/ml of hygromycin B to ensure the maintenance of the transfected plasmids. Aliquots of the clonal cells were stored in liquid N_2 and were thawed and expanded before experiments. The transfectants were seeded for subsequent experiments at a density of $3–6 \times 10^4$ cells/cm^2 in tissue culture plates and were used within 3–4 days after plating.

2.3. Relative quantitative RT-PCR

To determine the level of HSP70 transcripts, relative reverse transcription-polymerase chain reaction (relative RT-PCR) was performed. Total RNA (2.5 μg) was prepared from transfected cells and treated with DNase I. The RNAs were reverse transcribed into cDNAs at 42 °C for 60 min using a RETROscript firststrand synthesis RT-PCR kit (Ambion, Austin, TX). PCR was then performed in a reaction (10 μl) containing 1 μl RT product, 1 μl 10× PCR buffer (20 mM $MgCl_2$), 1 μl 2.5 mg/ml BSA, 1 μl 2 mM dNTP, 1 μl 10× LC Green I dye and 1 μl each of 5 μM forward and reverse primers. For rat HSC70 transcript, C73F forward (5′-TGATGAAGACAAACAGAAGA-3′) and C73R reverse primers (5′-TGAAGAAGCACCACCAGATG-3′) were used; the predicted RT-PCR product was 226 bp in length. For rat HSP70i transcript, P72F forward (5′-CTCGTGCGTGGGCGTGTTCC-3′) and P72R reverse primers (5′-TCGCCCTTGTAGTTCACCTG-3′) were employed. For human HSP70i transcript, H72F forward (5′-CTCCTGCGTGGGGGTGTTCC-3′) and H72R reverse primers (5′-TCCCCCTTGTAGCTCACCTG-3′) were used. Both rat- and human-specific HSP70i primer pairs generated a 285-bp fragment. As an internal control, the QuantumRNA™ 18S RNA-specific primers that generated a 324-bp fragment (Applied Biosystems/Ambion, Austin, TX) were included in the same multiplex-RT-PCR. The optimal 18S primer to competimer™ ratio was 4:6 for HSC70 and 2:8 for both rat and human HSP70i. The linearity of these reactions was predetermined; both HSC70 and HSP70i PCR reactions ran for a total of 27 cycles. The resultant PCR products were analyzed in a 6% native polyacrylamide gel and stained with Syber Gold (Molecular Probes, Eugene, OR). Data interpretation was done by dividing the signal obtained from either the HSC70 or HSP70i amplicon by the signal obtained from the 18S RNA amplicon.

2.4. Western blot analysis

Approximately 3 μg (to detect HSC70) or 8 μg (to detect HSP70i) of total proteins were separated by 7.5% SDS/PAGE. The resolved proteins were transferred to a Rad-Free membrane (Schleicher & Schuell, Keene, NH) using a Hoefer Blotter. The membrane was blocked with 5% BSA in PBS for 30 min and incubated overnight with an anti-HSC70 or -HSP70i monoclonal antibodies (SPA-815 and SPA-810, respectively, Enzo Life Sciences, Plymouth Meeting, PA) in PBS containing 0.4% NP-40. The membrane was rinsed in PBS containing 0.4% NP-40 and incubated with an alkaline phosphatase-conjugated secondary antibody for 3 h. Upon the completion of a color reaction in the presence of alkaline phosphatase substrates, the optical density of the HSC70 or HSP70i band was determined by densitometry. An identically loaded gel was prepared and stained with Coomassie Brilliant Blue R250. To adjust for loading differences, the optical density of the HSP70 band was normalized to that of the β-actin band on the Coomassie stained gel. To quantitate the level of HSP70i protein in heat preconditioned cells, signals detected in cell lysates derived at various time-points after heat preconditioning were compared to that generated from different dilutions of a purified HSP70 protein (SPP-755, Enzo Life Sciences, Plymouth Meeting, PA).

2.5. Thermal pretreatment

Culture plates were sealed with parafilm and immersed into a Precision shaker bath maintained at 43 + 0.1 °C for 20 min. Following heat pretreatment, cells were refed with fresh medium and returned to a CO_2 incubator at 37 °C. At designated time points, the

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