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The role of iHsp70 in myogenic stem cell resistance

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Various pathological processes caused by toxic environmental impacts negatively affect all types of cells including stem cells. Heat shock and other stresses are known to afford protection of various intracellular systems by inducing iHsp70 (inducible heat shock protein 70). The goal of this study was to investigate various ways of iHsp70 induction and its role in the viability of newly developed rabbit skeletal-muscle-derived stem cells. Skeletal myoblasts (Mio9), subjected to heat and chemical stresses, transiently or permanently transfected, significantly increased the intracellular amount of iHsp70 in rabbit skeletal myoblasts that correlated with increased cell resistance to secondary toxic exposures. The efficiency of iHsp70 induction has been proved using indirect immunocytochemistry, immunobloting and flow cytometry. Adult myogenic stem cells with increased iHsp70 content are a useful model to study the molecular mechanisms of cell resistance both *in vitro* and *in vivo*, improving the survival of grafted stem cells and the efficiency of cytotherapy.

Key words: stem cells, heat-shock proteins, viability, adult myogenic stem cells

Abbreviations: MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide; iHsp70, induced heat shock protein; Mio9, rabbit skeletal-muscle-derived stem cell line No 9.

INTRODUCTION

Hsp proteins are highly conserved proteins, and their major aim is to protect cells from lethal physical (heating), chemical or other damages [1]. Depending on their molecular mass, Hsp proteins have been classified into four main groups such as Hsp90, Hsp70, Hsp60 and Hsp27 [2]. Each Hsp family contains a constitutively or regularly expressed (Hsc) and stress-induced (Hsp) member [3]. The intracellular function of Hsp as molecular chaperones involves the protein folding / unfolding process, transport mechanism, repair and degradation of damaged proteins [4–6]. Responding to diverse stimuli, cells can rapidly express the induced form of heat shock proteins (iHsp), particularly iHsp70 [2]. Constitutively expressed proteins (Hsc) are abundantly expressed in cells and do not respond to extracellular stress [1]. The specificity of chaperone activity of Hsp depends on the structure of the chaperone, the size and location of the target proteins [2].

The major Hsp70 family consists of Hsc73 and Hsp72 members: the Hsc73 (Hsc70) form is constitutively expressed and encoded by one gene, whereas two genes encode the Hsp72 (Hsp70) form inducible by heat shock, oxidative stress, chemicals and various infections [3, 7]. All Hsp genes contain an ATP-ase domain, a peptide-binding domain, a nuclear location signal, and a carboxy-terminal domain [8]. The specificity of peptide binding determines different Hsp isophorms whose exact function in each case should be determined.

Recently it has been revealed that Hsp(s), besides its chaperonic function, regulates cell viability. Hsp(s) function in multiple cell viability monitoring pathways suggesting that a constant interaction between these pathways determines the fate of stressed cells [3, 9]. Abundant observations revealed that heat shock-induced Hsp72 (iHsp70) exert a significant multiple anti-apoptotic effect through the regulation of extrinsic and intrinsic apoptotic pathways [10–12].

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In this study, we have investigated the means of iHsp70 inducion in rabbit skeletal muscle-derived stem cells by heat and chemical stresses and genetical manipulations. All ways of iHsp70 induction showed an increased amount of intracellular iHsp70 identified by the immunocytochemical, immunoblotting and flow cytometry methods. The muscle-derived stem cells possessing iHsp70 demonstrated a higher resistance to secondary toxic exposures than the control cells. Increasing the resistance of muscle-derived stem cells through iHsp70 induction could be a shortest way to broaden stem cell usage in treating various diseases by medical stem cell transplantation.

MATERIALS AND METHODS

Cell culture and chemicals

Rabbit muscle-derived stem cells were isolated, cultivated and identified as previously described [13]. Cells were grown in 25 cm^3 polystyrene tissue culture flasks containing IMEM growth medium (Invitrogen) supplemented with FCS (10%), penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were kept at 37 °C in a humidified atmosphere with 5% CO_2 and passaged twice a week by detaching them from the flasks with 0.25% (w/v) trypsin / EDTA solution (Gibco, Grand Island, NY).

Monoclonal antibodies against the induced iHsp70 (iHsp72) and cognitive Hsc70 (Hsc73) forms of the Hsp70 group were purchased from the Stressgen Biotechnologies Corporation (Canada). Secondary anti-mouse and anti-rabbit antibodies were obtained from the New England Biolabs (UK).

Indirect immunochemistry

The procedure was done as written in [14]. Briefly, cells were seeded on cover slips in 24 well plates and grown overnight. Cells were washed with PBS, fixed in 4% PAF (paraformaldehyde), permeabilized with 0.1% Triton X-100 and blocked in 2% BSA, then incubated with a monoclonal Hsp70 antibody (Stressgen), washed with PBS and incubated with a secondary IgG antibody. The cover slips were mounted in the Citofluor mounting medium (Agar Scientific; Stanstead, UK). Cells were observed with a Nicon C1 confocal scanning microscope with controlled light exposure. The confocal image of cells was obtained with the PlanApo-VC immerse objective (magnification ×60, NA 1.4) and EZ-C1 programme. The Alexa 488 secondary monoclonal antibody was excited by the Ar(488 nm) laser, and emission was detected through a 522-nm filter. The obtained images were analyzed with the Bitplane programme Imaris 5.0.1. For each experiment an antibody control was done omitting the primary antibody from a sample and optimizing the specific fluorescence.

Flow cytometry assay

Cells transfected with pEYFP-Hsp70 plasmid were selected and grown with 100–200 μM G418. Cells had been freshly seeded 24 h before the experiment, then lifted with trypsin / EDTA solution, washed with PBS, and their fluorescence was analyzed with a BD FacsCanto II flow cytometer using an Ar (488 nm) laser for the excitation and the emission wavelength of 534 nm.

Induction of iHsp70 protein

hsp70 plasmids (pCMV70 and pEYFP-HSP70) were kindly provided by Dr. J. Hageman and Dr. J. Kampinga (Department of Radiation and Stress Cell Biology, University Medical Center Groningen, The Netherlands). The fluorescent plasmid (pEYFP-HSP70) was used for permanent transfection and had been constructed using the pEYFP-C1 vector expressing yellow fluorescent protein (YFP) from the CMV promoter together with the introduced *hsp70* gene [15]. The pCMV70 plasmid was used for temporal transfection and had been constructed using the pCMV5 vector [16]. The selection of resistant bacteria and eucariotic cells possessing *hsp70* gene was made using Canamycin A and Geneticin (G418), respectively. Cells had been seeded 24 hours before transfection. DNA (1 μg) was permanently transfected into the cells, using the polycations polybrene and lipofectamine (Invitrogen) according to the manufacturer's instructions. Temporal transfection was done using 10–20 μg of DNA, 0.25 M $CaCl_2$ and 2 × HEBS buffer (280 mM NaCI, 10 mM KCI, 1.5 mM Na_2HPO_4, 12 mM (d)-glucose, 50 mM HEPES, pH 7.1). DNA precipitates were added to the cells and incubated for additional 24–48 h.

The chemical induction of iHsp70 was done using the proteosomal inhibitor geldanamycin. Geldanamycin concentrations and incubation time are shown in Fig. 1 *A*.

For heat-shock experiments, rabbit muscle-derived adult stem cells were cultured in a complete medium at 37 °C overnight as described above. The medium was then changed, and the cells were heat-shocked in a 42 °C water bath for 15–60 min. At the end of the heat shock, plates were transferred back to the normal growth conditions in the 37 °C incubator. Control (not heat-shocked) cells were maintained in the same conditions.

The efficiency of transfected and stressed cells was revealed immunocytochemically, using immunobloting and flow cytometry. Data are presented as a representative experiment from at least three repeats.

Cell viability assessment

The cell viability test before and after iHsp70 induction was done with a trypan blue assay. Briefly, cells were lifted by trypsin / EDTA solution, washed in PBS and suspended in 0.4% trypan blue in PBS. Then the cells were counted with a

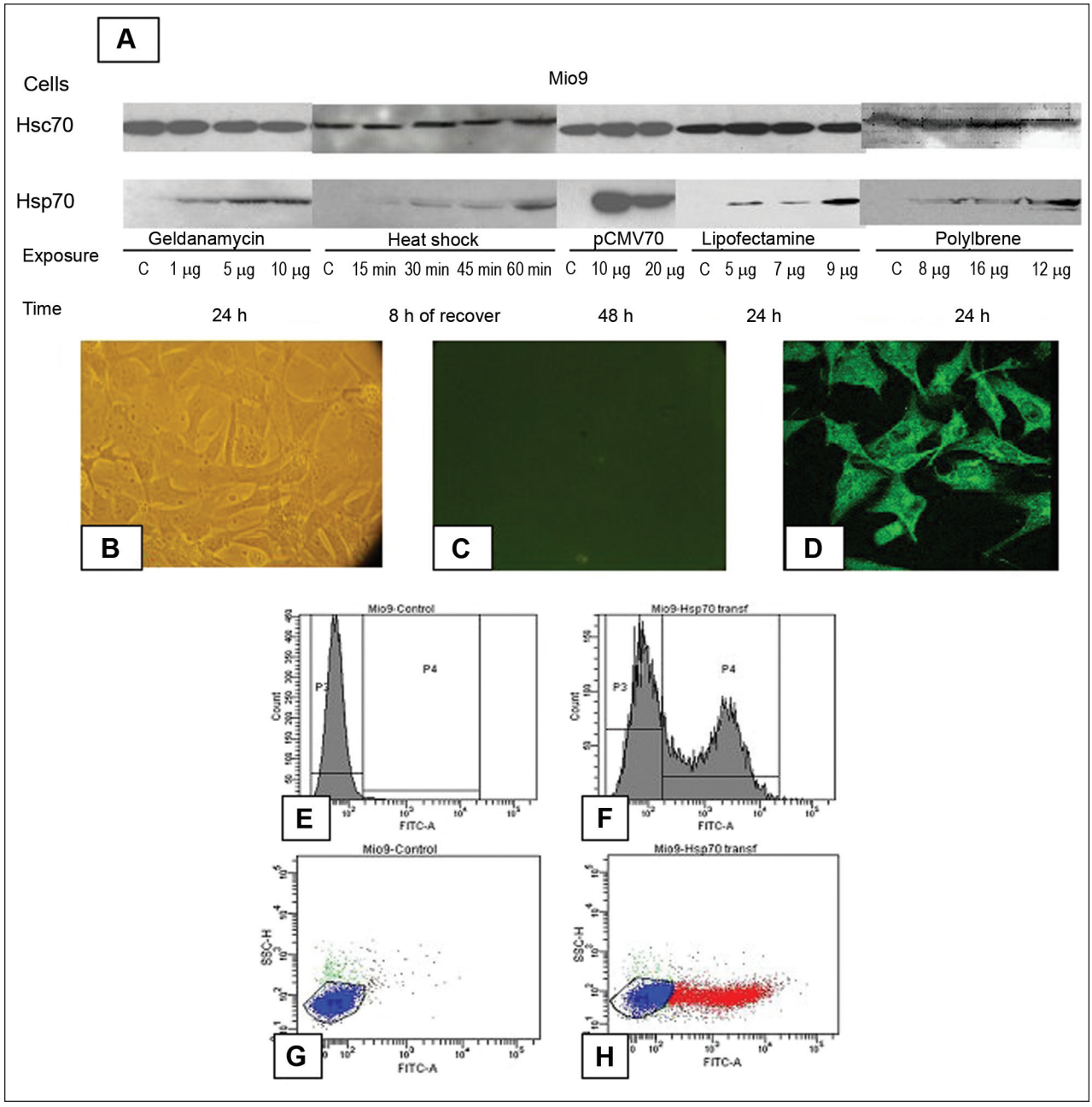

Fig. 1. Identification of iHsp70 induction in myogenic stem cells. A – immunoblotting of cells after heat and chemical stresses (geldanamycin), transient (10–20 µg of DNA, 0.25 mM $CaCl_2$) and permanent (1 µg of DNA, lipofectamine and polybrene) transfection; B – morphological analysis by light microscopy; C – indirect immunocytochemistry of control cells; D – indirect immunocytochemistry of transfected cells; E – flow cytometry histogram of control cells: P3 = 98.9 ± 0.8%, P4 = 1.1 ± 0.56%; F – flow cytometry histogram of transfected cells: P3 = 25.2 ± 2.5%, P4 = 74.8 ± 4.5%; G – flow cytometry dot plot of control cells; H – flow cytometry dot plot of transfected cells. One representative experiment of three is shown

hemocytometer: viable cells were colourless, whereas dead cells were coloured blue. Cell viability before and after iHsp70 induction was never below 95%.

The cytotoxicity test was done using MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) [17]. Briefly, the incubation medium after treatment with compounds was replaced with 0.5 ml of 0.2 mg/ml tetrazolium dye 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide, cells were incubated for 0.5 h at 37 °C, washed with PBS, formazan was extracted with ethanol and monitored at 570 nm. The mean OD_{570} of the cells with and without iHsp70, exposed to a test compound-free culture medium, was set to represent 100% of viability, and all other results were expressed as a percentage of these controls. Cells were exposed to various concentrations of compounds for 24 h.

Statistical analysis

Data are presented as a mean ± S.D. of at least three independent experiments. Statistical analyses were performed using Student's t test with the SigmaPlot 2001 programme. Results were considered statistically significant at $p < 0.05$.

RESULTS

Rabbit muscle-derived stem cells are fairly new and little studied stem cells. Our goal was to investigate various possible ways of iHsp70 induction and to study viability changes of myogenic stem cells after treatment with toxic compounds. Data of immunoblotting in Fig. 1 A show that iHsp70 induction in myogenic stem cells is feasible by chemical and heat stresses, temporal (using $CaCl_2$) and permanent (using lipofectamine and polybrene polycations) transfections with *hsp70* carrying plasmids as written in Materials and Methods. The iHsp70 expression after heat and chemical stresses, and temporal transfection additionally, were tested by indirect immunocytochemistry (Fig. 1 *C, D*). The morphology of transfected cells was analysed with a light microscope (Fig. 1 *B*) and did not show any changes. Indirect immunocytochemistry of control cells (Fig. 1 *C*) showed no presence of iHsp70. Control cells (Fig. 1 *E, G*) as well as cells transfected with fluorescent plasmid construct (Fig. 1 *F, H*) were tested by flow cytometry. The efficiency of iHsp70 induction was 70.0–80.0% (Fig. 1 *F, H*).

The viability of myogenic stem cells before and after iHsp70 induction was examined by the trypan blue extrusion test and was never below 95%, showing that iHsp70 induction is not harmful for a cell. The viability of control cells (without iHsp70) and of cells possessing iHsp70 after incubation with hydrogen peroxide (Fig. 2 *A*), 5,8-dihydroxy-1,4-naphthoquinone peroxide (Fig. 2 *B*), sodium nitropruside (Fig. 2 *C*), and daunorubicin (Fig. 2 *D*) was analysed by an MTT assay as written in Materials and Methods (Fig. 2). The cell protection was most efficient after exposure with

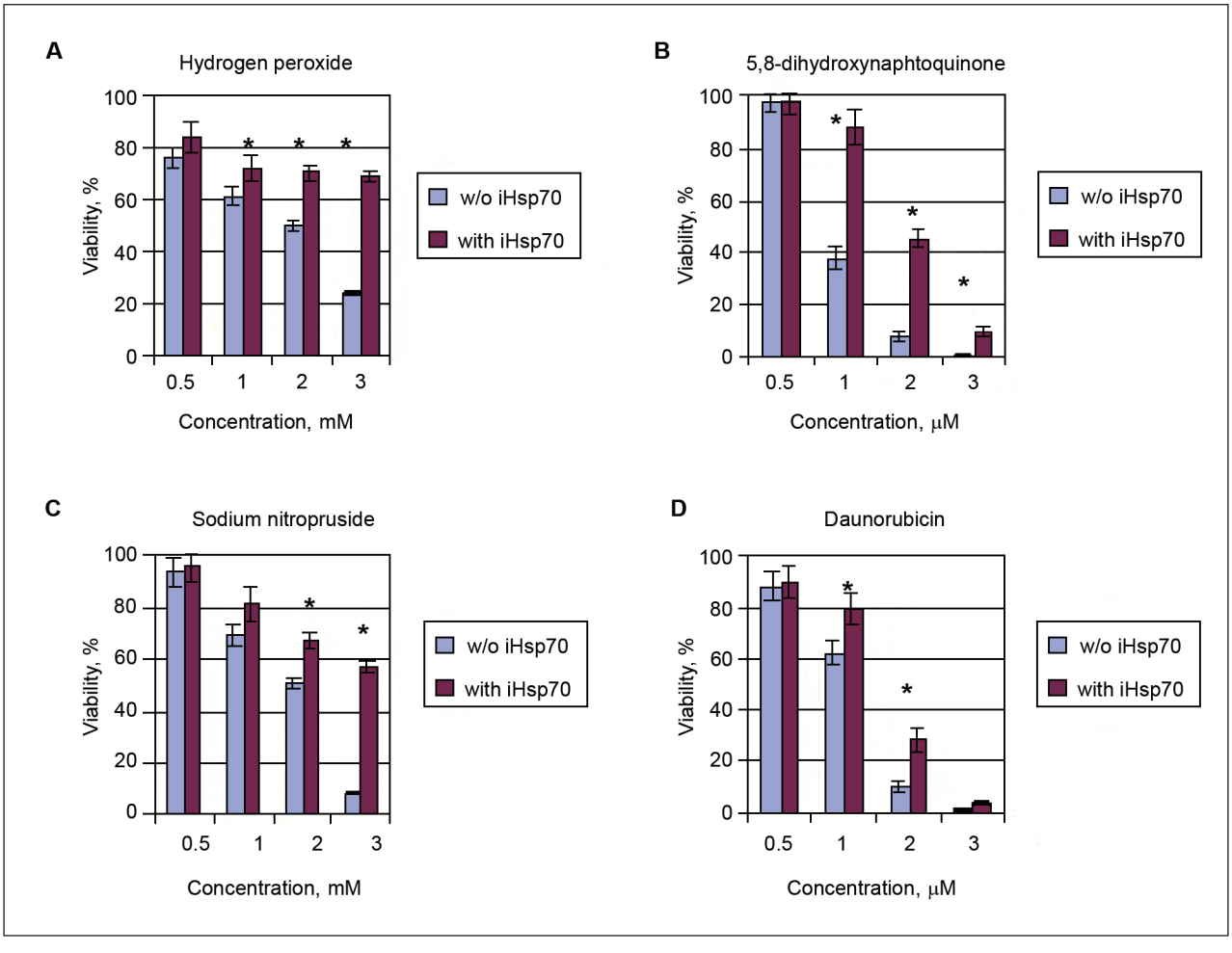

Fig. 2. Myogenic stem cell viability test. Cells with and without iHsp70 were exposed to various concentrations of hydrogen peroxide (A), 5,8-dihydroxy-1,4-naphoquinone (B), sodium nitropruside (C) and daunorubicin (D) for 24 h in normal growth conditions. Viability data are from at least three independent experiments and are presented as a percentage of absorption with control (compound-free culture medium exposed) cells ± SD. * – significant difference between cells with and without iHsp70, $p < 0.05$

high concentrations of hydrogen peroxide, 5,8-dihydroxy-1,4-naphtoquinone and sodium nitropruside (45–50%) (Fig. 2 *A–C*). Triplicate cell viability experiments ± S.D. are presented in Fig. 2. Our data have revealed that increased intracellular amounts of iHsp70 protect cells from secondary toxic exposures. The exact mechanisms by which iHsp70 increases myogenic stem cell resistance require more detailed studies.

DISCUSSION

Hsp70 can be induced by a variety of toxic environmental and pathophysiological conditions [1, 18]. Overexpression of Hsp70 has been reported not only to protect cells *in vitro* but also to enhance rat myocardial tolerance to ischemia-reperfussion [19, 20]. The iHsp70 can also protect cells from UVC irradiation-induced DNA damage as well as demonstrates an anti-apoptotic mode of action, involving a novel p53-inducible gene [21, 22]. The iHsp70 might repair DNA through excision repair enzymes or stimulate DNA polymerase beta gap-filling activity [23, 24]. On the other hand, iHsp70 is involved in cell protection by correcting protein folding and translocation through the membranes and the degradation of damaged proteins [25]. Data of this study show that iHsp70 could be induced in newly developed rabbit skeletal muscle-derived stem cells and be an *in vitro* model to study the molecular mechanisms of their increased resistance.

Skeletal myoblast transplantation is a promising way to treat the heart at the end-stage. Transplanted cells in an infarcted heart are subjected to a pathological environment causing stem cell death instead of adaptation and growth. The iHsp70 induced by a mild stress could be one of the progressive ways to increase the efficiency of using stem cells for transplantation. Studies performed on isolated neonatal and adult cardiomyocytes or cell lines showed a significant protective effect of iHsp70 [26]. It is also known that primer mild stress induces iHsp70 and increases body tolerance to high and normally lethal temperatures, ischemia or other kinds of stress [27, 28]. Increased Hsp70 and Hsp90 synthesis protects humans against myocardial infarction [29]. Similarly, Hsp70 protects neurones after stroke as well as tissues after transplantation [30, 31]. Hsp70 induction eases the consequences of chronic diseases such as diabetes [32]. Moreover, Hsp proteins also prevent Alzheimer's, Parkinson's and Huntington's neurodegenerative diseases [33–35]. Hsp proteins protects organs after various traumas as well as prevent cell ageing [36, 37]. In mice heart with the genetically induced high level of iHsp70, recovery after a short global ischemic stroke was faster and better [38].

Cell culture is a very useful model *in vitro* to study various intracellular processes including the induction and mode of action of heat shock proteins. Of course, studies performed on isolated adult stem cells should be interpreted with caution, because the cell growth conditions are unnatural and many environmental factors can influence cell response. Through scientific thick and thin, stem cell research, both *in vitro* and *in vivo* model systems, is moving ahead, answering many questions and making stem cell therapy safe and effective.

Data of this study demonstrate that iHsp70 in muscular stem cells can be induced by various techniques and all of them increase the intracellular amount of iHsp70, which correlates with an increased cell resistance to secondary toxic exposures. Our newly developed rabbit skeletal-muscle-derived stem cells might be a good model for studying the molecular mechanisms and regulation of stem cell death and survival processes. Induction of iHsp70 by various techniques can be a useful way to strengthen stem cells and increase the efficiency of stem cell therapy. This field is under investigation.

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References

1. Lindquist S, Craig EA. Annu Rev Genet 1988; 22: 631–77.
2. Hightower LE. Cell 1991; 66: 191–7.
3. Garrido C, Gurbuxani S, Ravagnan L, Kroemer G. Biochem Biophys Res Comm 2001; 286: 433–42.
4. Hartl FU. Nature 1996; 381: 571–80.
5. Ryan MT, Pfanner N. Adv Protein Chem 2001; 59: 223–42.
6. Kampinga HH. Handb Exp Pharmacol 2006; 172: 1–42.
7. Tavaria M, Gabriele T, Kola I, Anderson RL. Cell Stress & Chaperones 1996; 1: 23–8.
8. Zhu X, Zhao X, Burkholder WF et al. Science 1996; 272(5268): 1606–14.
9. Jaattela M. Ann Med 1999; 31: 261–71.
10. Beere HM, Wolf BB, Cain K et al. Nat Cell Biol 2000; 2: 469–75.

11. Beere HM, J Cell Sci 2004; 117: 2641–51.
12. Beere HM. J Clin Invest 2005; 115: 2633–9.
13. Bukelskienė V, Baltriukienė D, Bironaitė D et al. Seminars in Cardiology 2006; 11: 1–7.
14. Ohtsuka K, Laszlo A. Exp Cell Res 1992; 202: 507–18.
15. Hut HMJ, Kampinga HH, Sibon OCM. MBoC 2005; 16: 3776–85.
16. Michels AA, Kanon B, Konings AWT et al. J Biol Chem 1997; 272: 33283–9.
17. Mosman TJ. Immunol Meth 1983; 65: 55–63.
18. Macario AJ, Conway de Macario E. N Engl J Med 2005; 353: 1489–501.
19. Suzuki K, Murtuza B, Samut JA et al. Circulation 2002; 106(12): 1270–6.
20. Okubo S, Wildner O, Shah MR et al. Circulation 2001; 103(6): 877–81.
21. Niu P, Liu L, Gong Z et al. Cell Stress & Chaperones 2006; 11(2): 162–9.
22. Park WR, Nakamura Y. Cancer Res 2005; 65: 1197–206.
23. Kenny MK, Mendez F, Sandigursky M et al. J Biol Chem 2001; 276(12): 9532–6.
24. Mendez F, Kozin E, Bases R. Cell Stress & Chaperones 2003; 8: 153–61.
25. Nelson RJ, Ziegelhoffer T, Nicolet C et al. Cell 1992; 71: 97–105.
26. Mestril R, Giordano FJ, Conde AG, Dillmann WH. J Mol Cell Cardiol 1996; 28: 2351–8.
27. Murry CE, Jennings RB, Reimer KA. Circulation 1986; 74: 1124–36.
28. Marber MS, Mestril R, Chi S-H. J Clin Invest 1995; 95: 1446–56.
29. Marber MS, Latchman DS, Walker JM, Yellon DM. Circulation 1993; 88: 1264–72.
30. Yenari MA, Fink SL, Sun GH et al. Ann Neurol 1998; 44: 584–91.
31. Perdrizet GA, Kaneko H, Buckley TM et al. Transplant Proc 1993; 25: 1670–3.
32. Kurthy M, Mogyorosi T, Nagy K et al. Ann NY Acad Sci 2002; 967: 482–9.
33. Warrick JM, Chan HY, Gray-Board G et al. Nat Genet 1999: 23: 425–8.
34. Carimichael J, Chatellier J, Woolfson A et al. Proc Natl Acad Sci USA 2000: 97: 9701–5.
35. Sittler A, Lurz R, Lueder G et al. Hum Mol Genet 2001; 10: 1307–15.
36. Soti C, Csermely P. Exp Gerontol 2003; 38: 1037–40.
37. Kalmar B, Greensmith L, Malcangio M et al. Exp Neurol 2003; 184: 636–47.
38. Trost SU, Omens JH, Karlson WJ et al. JCI 1998; 101: 855–62.

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iHSP70 BALTYMO VAIDMUO DIDINANT MIOGENINIŲ KAMIENINIŲ LĄSTELIŲ ATSPARUMĄ

Santrauka

Įvairių toksinių aplinkos veiksnių sukelti patologiniai procesai neigiamai veikia įvairias ląsteles, tarp jų ir kamienines. Yra žinoma, kad tiek terminis, tiek ir kitoks stresas gali stimuliuoti iHsp70 (terminio šoko indukuotas baltymas; angl. *induced heat shock protein*) baltymo atsiradimą ir taip iš dalies apsaugoti ląsteles. Šio straipsnio tikslas buvo ištirti įvairius iHsp70 baltymo indukcijos būdus naujos rūšies kamieninėse ląstelėse – triušio skeleto raumens kamieninėse ląstelėse – sukėlus joms terminį bei cheminį stresą ir atlikus laikiną bei nuolatinę transfekciją dviejų rūšių plazmidėmis, taip pat ištirti ląstelių gyvybingumo pokyčius. Gauti duomenys rodo, kad įvairiais būdais padidintas iHsp70 kiekis triušio skeleto raumens kamieninėse ląstelėse gerino šių ląstelių atsparumą antriniam toksiniam poveikiui. iHsp70 sintezės efektyvumas buvo įrodytas imunoblotingo, imunocitochemijos ir tėkmės citometrijos metodais. Miogeninės kamieninės ląstelės, turinčios didesnį iHsp70 baltymo kiekį, gali būti puikus eksperimentinis modelis tiriant kamieninių ląstelių atsparumo mechanizmus tiek *in vitro,* tiek *in vivo* ir pagerinti transplantuotų kamieninių ląstelių išgyvenimą patologiniame židinyje, o kartu ir citoterapijos efektyvumą.

Raktažodžiai: kamieninės ląstelės, terminio šoko indukuotas baltymas, miogeninės kamieninės ląstelės