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Whole-body hyperthermia-induced thermotolerance is associated with the induction of Heat Shock Protein 70 in mice

Yueh-Tsu King, Chih-Sheng Lin, Jyh-Hung Lin and Wen-Chuan Lee*

Cardiovascular Research Center, Department of Comparative Medicine, Pig Research Institute Taiwan, PO Box 23, Chunan, Miaoli 35099, Taiwan, Republic of China

*Author for correspondence (e-mail: wen-chuan@mailcity.com)

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Summary

Molecular mechanisms of whole-body thermotolerance (WBT) in mammals have not been investigated thoroughly. The purpose of this study was to assess the induction of the 70 kDa heat shock protein (HSP70) and antioxidant enzyme activity in animal WBT, which was induced by whole-body hyperthermia (WBH) in mice. As a preconditioning treatment, WBH was applied to mice to induce WBT. Synthesis of inducible HSP70 (HSP70i) and quantification of its increased level in liver were investigated by one- and two-dimensional polyacrylamide gel electrophoresis and immunoblotting. HSP70i synthesis in mice liver was induced by non-lethal WBH (41 °C, 30 min). When compared to control animals, the level of liver HSP70i increased substantially (by 3.6-fold; $P<0.0001$). When exposed to 30 min of hyperthermia preconditioning, and after recovery for 48 h, the survival rate was 88.2 %, which was significantly higher than that of the control group (37.5 %; $P<0.01$). Moreover, the survival rate of animals subjected to preconditioning for 15 min was 72.2 %, which was also significantly higher than that of the control group ($P<0.05$). In contrast, the survival rate of animals subjected to preconditioning for 45 min was 63.5 %, which was not different from the control group. Nonetheless, the protection index of the group subjected to 15 min and 30 min of preconditioning was 1.93 and 2.37, respectively. Furthermore, to assess their contributions to WBT, the activities of antioxidant enzymes were also measured. After 48 h of recovery in preconditioned animals, hepatic antioxidant enzyme activities, including superoxide dismutase, catalase and glutathione peroxidase, had not changed significantly. To study the molecular mechanism of WBT, we successfully developed a mouse model and suggest that, rather than the activities of antioxidant enzymes, it is HSP70i that has a role to help animals survive during severe heat stress.

Key words: whole-body thermotolerance, heat shock protein 70, HSP70, antioxidant enzyme, preconditioning, mouse.

Introduction

Thermotolerance is a biological response which enables organisms to survive sub-lethal high temperatures prior to experiencing a non-lethal heat exposure (Field and Anderson, 1982). Many studies have documented this phenomenon in cultured cells and in animals other than mammals (Mizzen and Welch, 1988; Laszlo, 1988; Ulmasov et al., 1992; Kampinga, 1993; Theodorakis et al., 1999). In contrast, limited studies in rodents have revealed that a marked whole-body thermotolerance (WBT) can be induced by hyperthermia preconditioning (Kapp and Lord, 1983; Li et al., 1983; Weshler et al., 1984). The molecular mechanism of mammalian WBT, however, has not been investigated in detail.

Heat shock proteins (HSPs), recognized as molecular chaperones, are families of highly conservative stress proteins (Lindquist and Craig, 1988; Ellis and van der Vies, 1991; Hutter et al., 1996). The 70 kDa HSP family (HSP70) is categorized into constitutive and inducible forms (Lindquist and Craig, 1988), which contribute to stress tolerance by increasing the chaperone activity in the cytoplasm (Nollen et al., 1999). The inducible form of HSP70 (HSP70i) has been proposed as a predictor or indicator for thermotolerance at either the cell or animal level (Li and Mak, 1989; Flanagan et al., 1995). Intriguingly, the protective role of HSP70i in mammalian thermal death has yet to be determined.

Reactive oxygen species (ROS), which are postulated to be cellular toxicants (Gorman et al., 1999; Davidson et al., 1996), can be induced through hyperthermia (Flanagan et al., 1998). Moreover, it has been proposed that increased superoxide dismutase (SOD) activity contributes to cellular thermotolerance (Loven et al., 1985). Several studies have revealed that ROS induces HSP synthesis, which is critical for cellular thermotolerance development (Gorman et al., 1999; Wong et al., 1998; Ciacarra et al., 1994), but the role of antioxidant enzymes in WBT is controversial (Currie and Tanguay, 1991; Stears and Yellon, 1994; Joyeux et al., 1997), and their role in mouse WBT remains to be determined

further. The aim of this study was to explore the roles of HSP70i and the activities of antioxidant enzymes in mouse WBT.

Materials and methods

Animal source and care

Specific-pathogen-free ICR (Institute of Cancer Research, 1948, USA), male 8-week-old mice were purchased from National Laboratory Animal Breeding and Research Center (National Science Council, Taiwan, ROC). The animals were held in feeding boxes with a wood chip (Beta Chip, Northeastern Products Co., USA) flour bedding. Food and water were supplied *ad libitum.* Animal care and handling followed the guidelines of 'The Animal Protection Law' of the Republic of China and 'The Guide for the Care and Use of Laboratory Animals' (Derrell et al., 1996). Operations and post-treatment care of the animals were performed under the instruction of a qualified veterinarian.

Whole-body hyperthermia

Prior to the treatments, the animals were stabilized at room temperature (25±1 °C) for 7 days. Whole-body hyperthermia (WBH) was performed on a heating pad. Animals were anesthetized and maintained throughout the operation by injection of 2.5 % Avertin saline (20 μl g^{-1} body mass, Aldrich, USA). A 100 % stock of Avertin contains 1 g 2,2,2-tribromoethyl alcohol in 1 ml tert-amyl alcohol. The animals were taped onto the heating pad and a rectal thermostat probe was inserted (Harvard, USA). The temperature was maintained at 41±0.1 °C. To prevent any unnecessary heat injury to the cephalic organs, a cushion was placed under the head. During the operation, room temperature was maintained at 25±1 °C. The sham control group received Avertin treatment but no preheat treatment. Preconditioning treatments were administered for intervals of 15, 30 and 45 min. Following a 48 h recovery period, a lethal challenging dose (41 °C, for 60 min) was given. The survival rate (SR) of each treatment is defined as the number of animals surviving after hyperthermia challenge / number of animals before hyperthermia challenge. Whole-body thermotolerance (WBT) of animals was assessed with protection index (PI) defined as the SR of preconditioned animals after challenge / SR of non-preconditioned animals after challenge.

Gel electrophoresis and immunoblot analysis

To avoid blood contamination, liver tissues from both the control and heat-treated ICR mice were thoroughly washed with 0.9 % saline. Homogenization of the tissues was performed by a polytron (PT3100, Switzerland) in homogenization buffer (10 g sucrose, 4.0 mg pefablo SC, 0.5 mol l^{-1} Tris-HCl, pH 6.8, in 100 ml). The crude homogenates were then centrifuged at 12,000 ***g*** for 5 min at 4 °C (Kubota1720, Japan). The supernatants were collected for further experiments.

One-dimensional polyacrylamide gel electrophoresis (PAGE) and the immunoblot analysis were conducted as described previously (Lee et al., 1996). The supernatant of the tissue homogenate was lysed in a sample buffer (pH 6.8) containing 62.5 mmol l^{-1} Tris-HCl, 2 % sodium dodecylsufate (SDS), 5 % 2-mercaptoethanol, 10 % glycerol and 0.002 % Bromophenol Blue. The sample was boiled for 5 min, cooled in an ice bath and then centrifuged at 12,000 ***g*** for 3 min. Using bovine serum albumin as a standard, the protein concentration was determined (Lowry et al., 1951). Approximately 75 μg of liver samples were subjected to 9 % SDS-PAGE.

For immunoblotting analysis, protein bands in the slab gels were transferred to a nitrocellulose membrane (Hybond-C extra, Amersham, USA) by a semi-dry method (OWL Scientific Plastics, Cambridge, UK). The blot was incubated for 1 h in a 3 % gelatin solution (pH 7.4) containing 20 mmol l^{-1} Tris-HCl, 500 mmol l^{-1} NaCl, 0.05 % Tween 20 (TTBS) and then rinsed with TTBS. Subsequently, the membrane was incubated with rabbit anti-human HSP70 (Hsp72) polyclonal antibody (SPA-812, StressGen, Canada; diluted 1:1000 in TTBS containing 1 % gelatin) and against porcine HSP90 (diluted 1:1000 in TTBS containing 1 % gelatin) (Huang et al., 1999) for 1 h at room temperature. After three washes in TTBS, the membrane was reacted with goat-anti-rabbit antibody conjugated with alkaline phosphate (Sigma, USA; diluted 1:5000 in TTBS containing 1 % gelatin) for 1 h at room temperature. The membrane was rinsed three times with TTBS and developed within 3 min by an alkaline phosphate conjugate substrate kit (BioRad, USA) at room temperature. For further quantitative analysis, gel and immunoblot images were obtained using a densitometer equipped with ImageQuant (Molecular Dynamics, USA).

Two-dimensional (2D)-PAGE was performed as described previously (King et al., 2000). Approximately 300 μg of protein was loaded onto the isoelectrofocusing (IEF) gel and electrophoresized at 400 V for 16 h and then at 800 V for 1 h. Subsequently, the IEF gels were laid onto 9 % SDS-polyacrylamide slab gels with a 4.75 % stacking gel in the second dimension. The immunoblotting methods and gel imaging assessment were as described above.

Activity assays of antioxidant enzymes

The methods were conducted as described previously (Lin et al., 1997). Tissues were sliced into small pieces and thoroughly washed with a 50 mmol l^{-1} potassium phosphate buffer. Tissue homogenization was performed as described above and the supernatants were prepared for enzyme activity assays. Protein concentration was determined by the Lowry method (Lowry et al., 1951). Total superoxide dismutase (SOD) activity was determined by means of inhibition of pyrogallol autooxidation (Marklund and Marklund, 1974). At 420 nm, the optical density of the mixture was measured using a spectrophotometer (DU7500, Beckman, USA) at 25 °C for 5 min. ΔA_{420} values, which ranged from 0.12 to 0.35, were recorded by a constant, per minute relationship increase under normal conditions. Catalase (CAT) activity was obtained spectrophotometrically by measuring H_2O_2 decomposition at

Table 1. *Survival rates after recovery for 48 h from various durations of whole-body hyperthermia at 41 °C*

Duration (min)	Survival rate (%)
0	100
15	100
30	97.2±5.6
45	72.2±14.3**
60	36.1±14.0**

Values are means ± S.D. from four independent experiments (N=9 animals in each experiment).

**Differences in survival rates between groups of control (0) and animals treated at 41 °C for 45 min (or 41 °C for 60 min) were significantly different (P<0.01).

25 °C and at 240 nm (Aebi, 1983). The absorbancy decrease was recorded for 1 min: $\Delta A_{240}/\Delta t$=15 s^{-1} values ranged between 0.02 and 0.10. Glutathione peroxidase (GSPx) activity was assessed by the Flohe and Gunzler (1984) method with minor modification. To inhibit CAT activity, tert-butyl hydroperoxide was employed as a substrate, rather than H_2O_2, and 1 mmol l^{-1} sodium azide. Reaction rate was determined at 340 nm and 37 °C for 5 min. All enzyme activities are expressed in unit mg^{-1} of protein. One SOD activity unit is the prescribed amount of enzyme required to inhibit pyrogallol autooxidation by 50 %. 1 CAT unit decomposes 1 μmol of H_2O_2 min^{-1} at 25 °C. 1 GSPx unit results in 1 μmol of oxidized glutathione (GSH) min^{-1}.

Statistical analysis

The SAS GLM procedure was employed to analyze quantitative data (SAS Institute, 1989). Differences among groups were determined by the Duncan method. P values less than 0.05 were considered statistically significant.

Results

Level of HSP70i increased in mice by non-lethal hyperthermia

The survival rates at 48 h of recovery from various durations of WBH at 41 °C in ICR mice are shown in Table 1. The recovery period was fixed at 48 h, because the animals' physical status was insufficiently stable to determine survival rate and HSP70 level. The 48 h survival rate of the group heated for 30 min (97.2 %) did not differ significantly from that of the sham control (100 %). However, the survival rates after 45 min and 60 min of treatment were reduced to 72.2 % and 36.1 %, respectively, which differ significantly from that of the control (P<0.01). In subsequent experiments that assessed the relationship between HSP70i and WBT in mice, the heat dose given was based upon these reduced survival rates. Liver has been reported to be the most sensitive organ to synthesize HSP70 in response to hyperthermia (Flanagan et al., 1995; Manzerra et al., 1997; Schiaffonati et al., 1994). The results of immunoblotting analysis indicated that liver

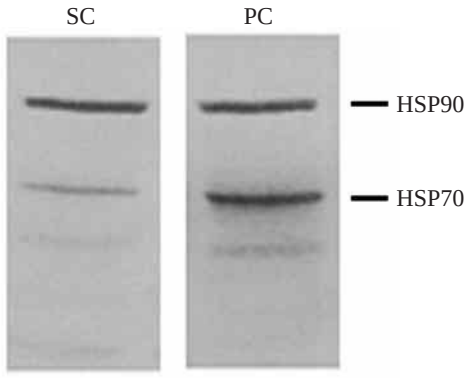

Fig. 1. A representative immunoblot of HSP70 in mouse liver. Mice were preconditioned at 41 °C for 30 min and then allowed to recover for 48 h. For one-dimensional gel electrophoresis, liver tissues of the mice from control (sham-treated; SC) or preconditioned (PC) groups were collected, processed and immunoblotted as described in Materials and methods. HSP90, HSP with a molecular mass of 90 kDa; HSP70, HSP with a molecular mass of 70 kDa.

HSP70 levels were increased substantially in preconditioned animals when compared to control (Fig. 1). From 2D-PAGE and subsequent immunoblotting, a spot with molecular mass 70 kDa and isoelectric point (pI) 5.25, characteristic of inducible HSP70 (HSP70i), was the most highly induced member of the HSP family in the heated mice (Fig. 2). Quantitatively, the HSP70i levels in the livers of the heated animals increased 3.6-fold after a 48 h recovery (P<0.01; Fig. 3).

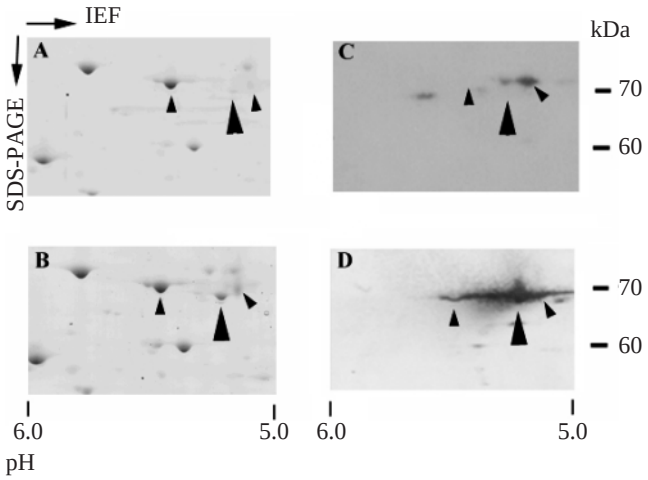

Fig. 2. Verification of HSP70 by 2D-PAGE and immunoblot analysis in mouse liver. Liver tissues from control (A,C) and preconditioned (B,D) mice were the same as those described in Fig. 1. Gel electrophoresis and immunoblotting are described in Materials and methods. Commassie Blue-stained gels (A,B) and immunoblots for HSP70i (C,D) are shown. Larger arrowheads indicate the HSP70i locations; smaller arrowheads indicate other members of the HSP70 family. IEF, isoelectrofocusing; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Molecular mass of the protein spots (in kDa) and the pH value for the range of IEF gels are indicated to the right and bottom, respectively.

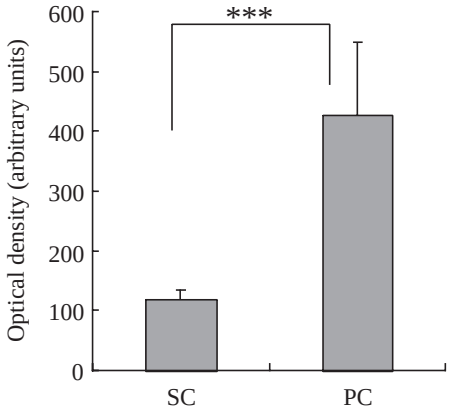

Fig. 3. Quantitation of HSP70i level in mouse liver. Liver tissue from control (sham-treated, SC; *N*=8) and preconditioned (PC; *N*=12) mice were the same as those described in Fig. 1. The procedures for performing immunoblotting and quantification of the HSP70i levels are described in Materials and methods. Levels of HSP70i in control and preconditioned tissues were significantly different ($P<0.0001$).

Whole-body thermotolerance is increased in hyperthermia-preconditioned mice

Based on the results described above, the preconditioning heat dose (41 °C for 30 min) and the challenging dose (41 °C for 60 min) were chosen to assess whether mice demonstrate WBT. The results of increased survival rates from WBH episodes at 41 °C for 30 min and 60 min are shown in Table 2. The survival rate after 48 h of recovery of the challenged ICR mice subjected to 30 min of preconditioning was 88.2 %, which was significantly higher than that of the control group (37.5 %; $P<0.01$). Moreover, the survival rate of animals subjected to 15 min of preconditioning was 72.2 %, which was also significantly higher than that of the control group ($P<0.05$). By contrast, the survival rate for mice subjected to 45 min of preconditioning was 63.5 %, which was not significantly different from that of the control group. Further, the protection index of the 15 min and 30 min preconditioning group was 1.93 and 2.37, respectively (Table 2). Therefore, WBT can be induced by hyperthermia preconditioning in mice.

Table 2. *Survival rates and protection index after recovery for 48 h following whole-body hyperthermia in preconditioned mice*

Preconditioning dose	Challenging dose	Survival rate	Protection index
0	41 °C, 60 min	37.5±17.8	1
41 °C, 15 min	41 °C, 60 min	72.2±6.4*	1.93
41 °C, 30 min	41 °C, 60 min	88.2±13.7**	2.37
41 °C, 45 min	41 °C, 60 min	63.5±22.7	1.26

Values are means ± S.D. from four independent experiments (*N*=9 animals in each experiment).

Survival rates in control and various hyperthermia preconditioned groups were *significantly different ($P<0.05$) or **very significantly different ($P<0.01$).

Table 3. *Activities of liver catalase, superoxide dismutase and glutathione peroxidase of control and preconditioned mice after recovery for 48 h*

Experiment	Enzyme activity (units mg^{-1} protein)		
	CAT*	SOD‡	GSPx§
Control (*N*=10)	276.8±50.1	7.1±1.8	3.1±0.5
PC (*N*=13)	252.8±82.0	8.0±2.4	3.1±0.6

PC, preconditioned.

*CAT (catalase), 1 unit decomposes 1 μmol H_2O_2 min^{-1} at 25 °C.

‡SOD (superoxide dismutase), 1 unit of enzyme activity is defined as the amount of enzyme that inhibits the autooxidation of pyrogallol by 50 %.

§GSPx (glutathione peroxidase), 1 unit produces 1 μmol oxidized glutathione min^{-1}.

CAT, SOD and GSPx activities in thermotolerant mice

Given the controversy associated with antioxidant enzymes in animal WBH (Currie and Tanguay, 1991; Stears and Yellon, 1994; Joyeux, 1997), CAT, SOD and GSPx activities were measured to assess their roles in thermotolerant mice. When compared to the control group, CAT, SOD and GSPx activities in liver of WBT group remained unaltered (Table 3).

Discussion

At the cellular level, the molecular mechanisms of thermotolerance have been investigated intensively. Some small HSPs (sHSP) such as HSP27 and HSP32 were reported to confer stress tolerance on animals or cells. HSP27 behaves as a microfilament protector (Landry and Huot, 1995; Lavoie et al., 1995) and HSP32, also known as heme oxygenase-1, can be upregulated by antioxidant pyrrolidinedithiocarbamate (PDTC), together with HSP70 (Stuhlmeier, 2000). However, reports indicate that cell content, including translation activity and cytoskeletal integrity, as well as cell survival, benefit from prior hyperthermia treatments in which HSP70 is the major overexpressed product (Nollen et al., 1999; Wong et al., 1998). The role of HSP70 in thermal resistance is also implicated at the whole animal level (Feder and Hofmann, 1999). However, the protective mechanism of HSP70 on WBT in mammals required further investigation. We hypothesized that, in various organs, a non-lethal WBH preconditioning induces various physiological responses, which in turn trigger HSP70 synthesis within cells, thereby protecting animals from thermal death. Physiological responses of WBH include alterations to mammalian cardiovascular, endocrine, and nervous systems (Hutter et al., 1996; Bull et al., 1982). Furthermore, the factors released from the responding tissues may contain stress hormones, cytokines and electrolytes, and reduced oxygen partial pressure, all of which induce HSP70 synthesis *in vitro* or *in vivo* (Bicher, 1980; Katschinski et al., 1999; Blake et al.,

1993). Therefore, the protective contribution of HSP70 to the cellular thermotolerance of various organs is acknowledged (Kampinga, 1993; Theodorakis et al., 1999; Flanagan et al., 1995).

However, to confer WBT, molecules other than HSP70 or stress proteins may also perform a function *via* different mechanisms. A cellular study has indicated that, after heat exposure, SOD activity increased (Loven et al., 1985). It is assumed that this increase coincided with HSP synthesis, as well as the thermotolerance development, which therefore suggests that SOD protects cells from heat stress (Gorman et al., 1999). Moreover, HL-60 cells are partially protected from hyperthermia-induced apoptosis, while incubated with exogenous CAT during heat exposure (Bicher, 1980). Furthermore, overexpressed GSPx in human MCF-7 cells increases anti-oxidative stress ability (Doroshow, 1995). Due to the compensation of other antioxidant enzymes, GSPx-deficient mice continue to survive normally (Ho et al., 1997). However, data presented here show that the residual activities of SOD, CAT and GSPx are not altered, whereas that of HSP70i is (Table 3). The anti-heat-stress effect of antioxidant enzymes on WBT therefore requires further investigation.

In this study we have clearly demonstrated that, in mice, thermotolerance is substantially induced by WBH. Moreover, we have demonstrated that the levels of HSP70i in these mice can be increased. We did not find any contribution of antioxidant enzymes in this animal model and, therefore, we conclude that, in mice, WBT increased by WBH is associated with HSP70i and not with CAT, SOD or GSPx activities, which enables the animal to survive an acute heat stress.

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