Journal of Cellular Biochemistry 79:453–459 (2000)

Cytoprotection by Electromagnetic Field-Induced hsp70: A Model for Clinical Application

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Abstract A unique approach to clinical application of cytoprotection is offered by electromagnetic (EM) field induction of stress proteins. EM fields are noninvasive and easily applied, as compared with the current hyperthermia protocols. Fertilized dipteran eggs and cultured rodent cardiomyocytes (H9c2 cells) were used as models to test EM fields for their ability to induce increased hsp70 levels for effective cytoprotection. Eggs preconditioned with an 8μT 60Hz EM field for 30 min had 114% increase in hsp70 levels, and an average 82% increase in survival, following a lethal temperature of 36.5°C. Thermal preconditioning at 32°C was not nearly as effective in dipteran eggs, inducing only a 44% increase in survival. Preconditioning of cultured murine cardiomyocytes (H9c2 cells) with an 8μT 60 Hz field induced a 77% average increase in hsp70 levels. J. Cell. Biochem. 79:453–459, 2000. © 2000 Wiley-Liss, Inc.

Key words: cytoprotection; 60Hz (60 cycles/sec); EM fields; hsp70 protein; HSP70 promoter; heat shock

There is increasing evidence that stress proteins/chaperones play crucial and protective roles in a variety of cellular processes, including repair or degradation of damaged proteins, and contribute to cytoprotection [Welch and Brown, 1996]. As the role of cytoprotective chaperones in cardioprotection in ischemic heart disease has become more widely understood, their induction prior to surgery has become more prevalent. To induce these proteins, clinical procedures have utilized whole-body hyperthermia in an effort to protect the mycardium during reperfusion ischemic stress following stroke and heart attack [Benjamin and McMillan, 1998]. However, this approach is resisted by the body, is cumbersome, difficult to administer with precision, and decidedly uncomfortable for the patient. A benign, noninvasive method for inducing increased levels of the stress protein hsp70 would be brief exposures to low-energy, low-frequency EM fields [Goodman and Blank, 1998]. The advantages of EM fields over hyperthermia include:

- low frequency EM fields, unlike high temperatures, penetrate all cells essentially without attenuation [Blank and Goodman, 1999].
- EM fields and heat induce stress proteins at very different energy input levels; the energy density required for a 0.8μT EM field to induce increased hsp70 levels is 14 orders of magnitude lower than the temperature rise (5.5°C) required for a comparable effect [Goodman and Blank, 1998].
- Cells respond rapidly to EM fields: exposure for only 10 s elicits a measurable response [Litovitz et al., 1991].
- Significant levels of hsp70 are induced well within 30 min from onset of EM field exposure [Goodman and Henderson, 1988; Blank et al., 1994] and the levels remain elevated for more than 3 h [Han et al., 1998].
- Unlike thermally induced (heat shock) stress proteins, EM field-induced protein levels can be restimulated with *different* field strengths (higher or lower) to even greater hsp70 levels [Goodman and Blank, 1998; Han et al., 1998].

Abbreviations used: EM, electromagnetic; hsp, heat shock protein; milligauss, mG; microtesla, μT (1μT = 10mG); HS, heat shock.

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Received 23 November 1999; Accepted 4 April 2000

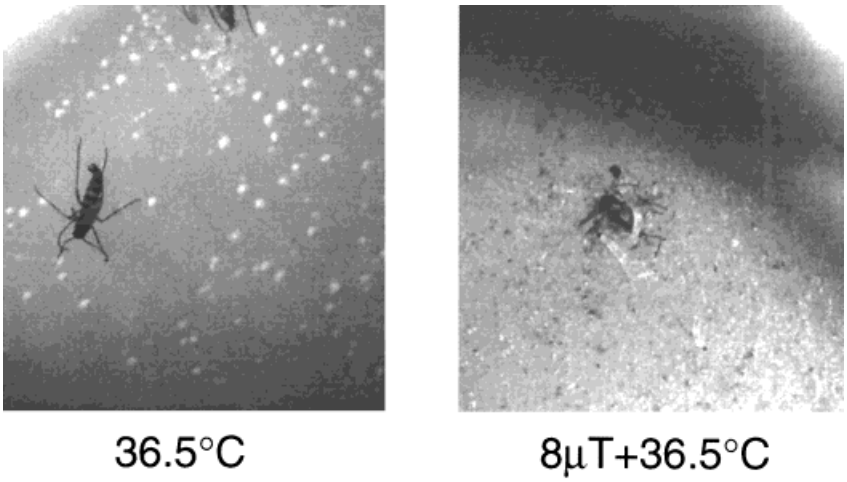

Fig. 1. Photographs of larvae from preconditioned eggs vs. eggs not preconditioned. **Left:** Eggs (white dots) that were subjected to lethal heat shock at 36.5°C without prior conditioning by EM fields, and that did not hatch into larvae. **Right:** Moving larvae (small black dots) that hatched from preconditioned eggs (8μT EM field) exposed to lethal heat shock at 36.5°C.

- EM field-induced protection will occur even if the field is applied immediately after the start of hypoxia, a condition that mimics treatment following onset of heart attack [DiCarlo et al., 1999].

The induction of these protective proteins by EM fields provides significant advantages in ease and simplicity of application for both patient and clinician and at the same time offers cardioprotection equivalent to hyperthermia. Since the ideal preconditioning agent would be noninvasive and easily administered in precise doses, the potential of stress protein induction by EM fields is medically attractive. The therapeutic use of EM fields is not new; EM fields have been used since the 1970s for healing bone non-unions and wound healing, and for the reduction of inflammation [Bassett, 1995].

To develop a protocol for EM field induction of hsp70 for cytoprotection, we selected fertilized dipteran eggs as a first-level model. Our rationale was that this would lead to an experimental protocol with a mouse infarction model. In some experiments we compared EM preconditioning induction of hsp70 levels in fertilized eggs (an in vivo model) with EM preconditioning induction of hsp70 in cultured rat cardiomyocytes (an in vitro model).

MATERIALS AND METHODS

Growth and Maintenance of *Sciara Coprophila*

Conditions for growth and maintenance of *Sciara coprophila* have been extensively described [Gabrusewycz-Garcia, 1964; Crouse, 1977; Zegarelli-Schmidt and Goodman, 1981]. For the purposes of this study, *Sciara* provided several distinct advantages over the more well-known dipteran model, *Drosophila*. Each *Sciara* female deposits approximately 75 eggs simultaneously four days after mating. In *Drosophila*, smaller numbers of eggs are deposited, and over a wider time frame. With appropriate matings it can be predetermined whether deposited eggs hatch into all female or all male adult flies using the wing marker, 'curly', that is phenotypic evidence of the presence of a gene that determines that eggs deposited by female flies carrying this wing marker will hatch into all female adult flies.

Rodent Cardiomyocytes

Cardiomyocytes (ATCC; H9c2) were grown in DMEM and maintained as indicated by American Type Culture Collection. Cells were treated with EM fields or heat at subconfluency, 1×10^6 cells/ml, in 15mls in Petri dishes (100mm).

Establishing EM and Temperature Pretreatment Conditions

To determine optimum field strength for survival and hsp70 induction, dipteran eggs (and cardiomyocytes) were exposed to field strengths of 0.8, 8, and 80μT at a frequency of 60Hz; 8μT was selected as a preconditioning field strength based on percent survival following a lethal temperature as well as the level of hsp70 induced (Figs. 1, 2).

To determine lethal and sublethal temperatures, eggs were exposed to temperatures from 30°C to 39°C; 36.5°C was the lethal temperature and 32°C the sublethal temperature. Sub-

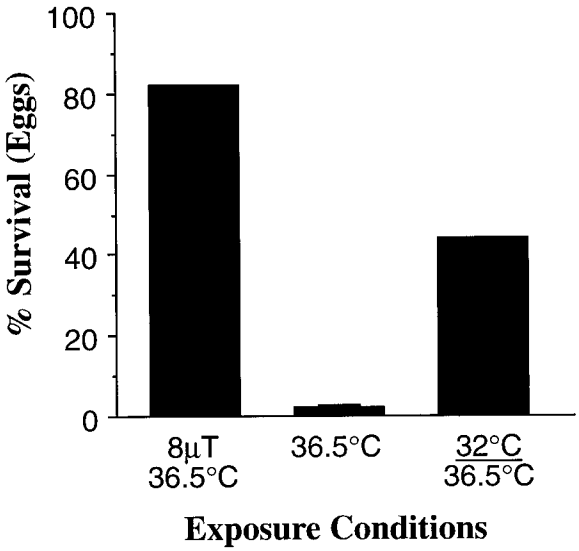

Fig. 2. Percent survival of fertilized dipteran eggs. **Lane 1.** Percent survival of eggs preconditioned with an 8μT EM field (30 min) followed by exposure to a lethal temperature (36.5°C for 60 min) (82% ± 0.16). **Lane 2.** Percent survival of eggs exposed 60 min to a lethal temperature (36.5°C) (2% ± 0.56). **Lane 3.** Percent survival of eggs thermally preconditioned (32°C) followed by exposure to a lethal temperature (36.5°C) (44%± 0.01). Data are the averages from 12 experiments (±standard error of the mean).

lethality was based on survival of eggs hatching into larvae after 60 min of exposure to a lethal temperature of 36.5°C. Heat shock for cardiomyocytes was 43°C.

Temperature Control

Temperature was monitored with a Physitemp (BAT-12) thermocouple probe (Physi-Temp, Hackensack, NJ) (sensitive to ± 0.1°C) to ensure that no heating resulted from the Helmholtz coils. The thermocouple probe was attached to the Helmholtz coils throughout all EM field exposures.

Heat Shock

Petri dishes containing the eggs or cardiomyocytes were wrapped in Parafilm, placed in mu metal containers and immersed in a water bath. Mu metal (see description below) shields against stray EM fields, in this case the fields generated by the heating unit in the water bath.

Conditions for Collecting Dipteran Eggs

Four females (curly winged) and four males were mated in each 100 mm Petri dish containing a 1 cm layer of 4% agar. Eggs were counted four days after mating and the number of eggs recorded. An accurate egg count was obtained using a 1.5 × 1.5 cm grid on the bottom of each Petri dish.

Protocol for EM Field and Thermal Conditions for Eggs

Petri dishes containing eggs were grouped as: (1) preconditioned with 8μT 60Hz EM fields for 30 min at 20°C followed by recovery for an additional 30 min. Eggs were then exposed to 36.5°C for 60 min and returned to the 20°C incubator; (2) eggs with no EM field preconditioning and exposed for 60 min to a lethal temperature (36.5°C) served as controls for group (1); (3) preconditioned with 32°C heat for 30 min followed by a 30-min recovery at room temperature. Eggs were then exposed to a lethal temperature of 36.5°C for 60 min and returned to a 20°C incubator; (4) eggs with no heat preconditioning and exposed to a lethal temperature (36.5°C) served as controls for group (3).

Protocol for EM Field and Thermal Conditions for Cardiomycocytes

Petri dishes containing cells (1×10^6cells/ml in 15 mls) were grouped as: (1) preconditioned: 8μT EM fields for 30 min at 37°C followed by recovery for an additional 30 min (and protein extracted); (2) 8μT EM preconditioned cells exposed to 43°C for 60 min (and protein extracted); (3) no preconditioning (and protein extracted); (4) no preconditioning and exposed to a 43°C lethal temperature for 30 min (protein extracted).

Determination of Percent Survival for Dipteran Eggs

Seven days following experimental or control treatment, Petri dishes were examined and *moving* larvae were counted and recorded (Fig. 1). The number of surviving larvae was compared to the initial number of eggs in each dish and survival percentages calculated.

EM Field Exposures

Two fully functional exposure units provided simultaneous sham and experimental exposures. Exposures used Helmholtz coils (Electric Research and Management, Pittsburgh, PA) that consisted of 19-gauge wire bundles wound

164 times around a form 13 cm × 14 cm with 8 cm spacing. The coils were energized by a function generator (11MHz Wavetek Stabilized Function Generator; model 21). A digital multimeter was used to measure the field intensity and verify the system's operation (Fluke 87 digital multimeter). Field parameters were monitored with a Hitachi V-1065 100MHz oscilloscope and calibrated inductive search coil (25×; Electro-Biology, Inc., Parsippany, NJ). Detailed description of the exposure system, including background magnetic fields in the incubator, harmonic distortion, DC magnetic fields and mean static magnetic fields in the incubator, both vertical and horizontal components, can be found in Jin et al. [1997]. Cells were placed on a Plexiglas stand in a horizontal orientation; that is, the entire area of the dish was exposed to the field. The bottom of the dish was 2 cm below the axis level. The height from dish bottom to top surface of liquid or the agar was approximately 1.1cm; the height of the liquid or agar was 0.6 cm. The calculated electric field was ≈11μV/m for an 8μT exposure.

Mu Metal Shielding

Helmholtz coils were enclosed within Mu metal containers to prevent Petri dishes containing cardiomyocytes and eggs from stray fields during all EM field exposures. Both active (experimental) and sham-exposed coils (controls) were enclosed in a 30 cm high, 15 cm diameter cylindrical mu metal container (.040″ thickness) (Amuneal Corp., Philadelphia, PA). The 60Hz shielding factor is (Min.) 90.1 (39.08dB). Sham-exposed controls and experimental exposures are performed simultaneously in identical mu metal containers.

Protein Lysates

Lysates were prepared from H9c2 cardiomyocyte cells as previously described [Lin et al., 1997; modification of Mosser et al., 1988]. Extraction of protein from eggs was accomplished by treating the eggs alternately with dry ice (30 min) and room temperature water (60 min) for 2 h together with glass beads. Protein concentrations were determined with a Bio-Rad protein assay kit (Bio-Rad Laboratories).

Western Blot

Protein lysates from dipteran eggs and cardiomyocytes were analyzed for hsp70 levels using the ECL detection system (Amersham). The intensity of the signal was determined with a PhosphorImager 400A (Molecular Dynamics) and quantified using ImageQuant software.

Antibodies

The human anti-hsp70 was supplied by StresGen; the Drosophila anti-hsp70.1 was generously provided by Dr. Susan Lindquist, University of Chicago.

Statistical Analyses

A sufficient number of experiments were performed to assure statistical significance. Samples from each experiment were tested three times and experiments repeated a minimum of six times. The data were entered into Excel for analysis and the results examined with a two-tailed t-test. Statistical significance is determined by multifactor analysis of variance program (INSTAT). The data is expressed as the ratio of the experimental to the control (E/C) ± standard error of the mean.

RESULTS

EM Field Preconditioning Induces Higher Survival Than Thermal Preconditioning

To determine percent survival, the number of eggs that hatched into moving feeding larvae 7 days after exposure to 36.5°C was divided by the initial number of preconditioned eggs (Fig. 1). Figure 2 shows that *Sciara* eggs preconditioned with an 8μT EM field for 30 min had an 82% increase in survival at a temperature of 36.5°C as compared with a 2.5% survival of eggs that were *not* preconditioned. Eggs thermally preconditioned at 32°C had a 44% increase in survival at 36.5°C.

Hsp70 Levels Induced by EM Fields in Dipteran Eggs and Rodent Cardiomyocytes

Figure 3 shows an example of hsp70 levels as determined by Western blot. Eggs pretreated with an 8μT EM field showed a 114% increase in hsp70 levels (lane 2). Eggs pretreated with an EM field and exposed to 36.5°C had a similar increase in hsp70, 110% (lane 4). There was a 12% increase in hsp70 in eggs heat shocked at 36.5°C (lane 3). Hsp70 levels in eggs with no pre- or post-treatment (controls) were lower than 5% (lane 1).

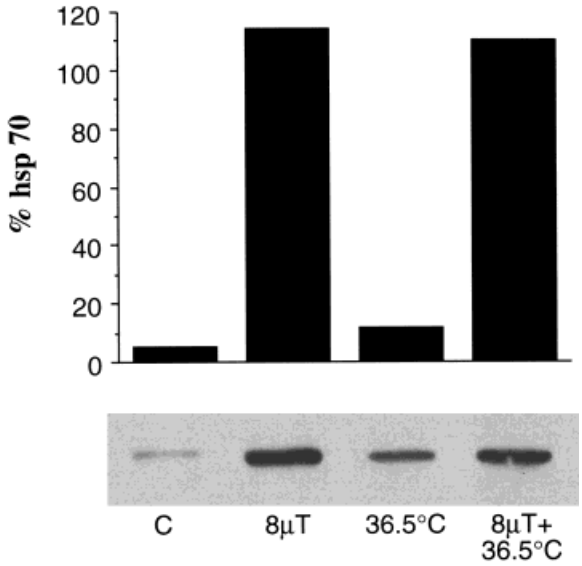

Fig. 3. hsp70 levels in dipteran eggs with and without EM field preconditioning.**Lane 1:** hsp70 levels in unpreconditioned eggs (controls); (<5%). **Lane 2:** hsp70 levels in eggs preconditioned with an 8μT EM field; (114%). **Lane 3:** hsp70 levels in unpreconditioned eggs exposed to heat shock (36.5°C); (12%). **Lane 4:** hsp70 levels in eggs preconditioned with an 8μT EM field followed by exposure to heat shock (36.5°C); (110%).

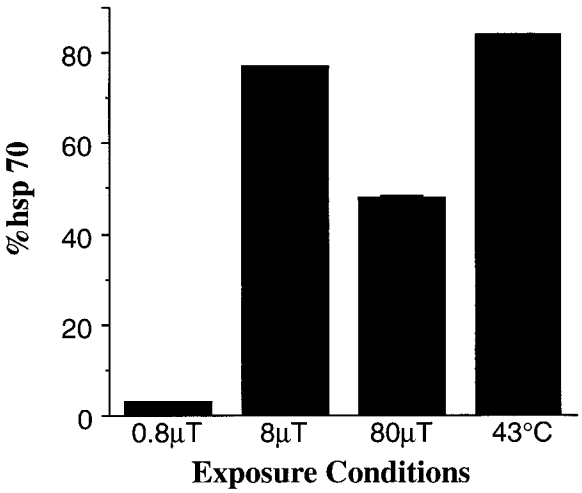

Fig. 4. hsp70 levels in cardiomycotes exposed to EM fields and heat shock. **Lane 1:** hsp70 in cells exposed to 0.8μT = 2.98% ± 0.08. **Lane 2:** hsp70 in cells exposed to 8μT = 77% ± 0.12. **Lane 3:** hsp70 in cells exposed to 80μT = 48%± 0.31. **Lane 4:** hsp70 in cells exposed to 43°C = 83% ± 0.31. Data averaged from eight Western blots.

Figure 4 shows the average levels of hsp70 in cultured cardiomyocytes exposed to three different EM field strengths; exposure to an 8μT EM field increased hsp70 levels 77% (±.12%), whereas exposure to an 80μT EM field increased hsp70 levels only 48% (±.31%). Exposure to an 0.8μT field induced virtually no increase in hsp70, only 2.9%. Cells heat shocked at 43°C showed an 83% (±.31%) increase in hsp70. An example of a Western blot from these experiments is presented in Figure 5.

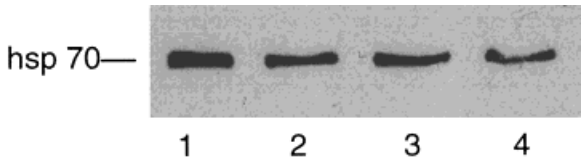

Fig. 5. hsp70 levels in cardiomyocytes exposed to EM fields and/or heat shock. **Lane 1:** Protein from cells exposed to heat shock at 43°C. **Lane 2:** Protein from cells exposed to an 8μT EM field followed by heat shock. **Lane 3:** Protein from cells exposed to an 8μT EM field. **Lane 4:** Protein from sham-exposed cells.

DISCUSSION

Based on the results from the experiments described here, we propose that the clinical benefits due to induced increases in hsp70 levels can be achieved more effectively by exposure to EM fields as by hyperthermia. Although our studies have focused on the major stress protein hsp70, EM fields induce increased levels of hsp27 and 90 as well [Han et al., 1998]. As in 'heat shock', EM fields induce activation of the HSP70 gene expression through trimerization of heat shock factor 1 (HSF1) and HSF1 binding to a heat shock element (HSE) in the HSP70 promoter [Lin et al., 1997, 1998]. Similar to 'heat shock', there is an EM field domain in the promoter region of the HSP70 gene, with a well-defined response element that differs from the 'heat shock' domain. Deletion of the EM field response element results in a 100% reduction in the EM field induction of hsp70 [Lin et al., 1999].

Of particular interest in terms of clinical application is that the increase in hsp70 protein levels induced by a 30-min EM field exposure persists for more than 3 h, and that a 30-min restimulation with a different (higher or lower) EM field strength at any time during this 3 h period induced even *higher* levels of hsp70 [Han et al., 1998].

Furthermore, there are detrimental side effects associated with 'heat shock' and its clinical application, hyperthermia, that are avoided by the use of EM fields:

- the messenger RNAs for basal (nonessential) cellular proteins, normally inhibited by 'heat

shock', are not affected by EM field-induced stress.
- although 'heat shock' is effective with cells and isolated tissues, hyperthermia has serious limitations when applied to human patients due to normal resistance by the body's thermoregulatory mechanisms.

Cardiovascular Cytoprotection With Heat-Induced hsp70

The induction of hsp70 for clinical application is a rapidly growing area of experimental medical research. Transgenic mice overexpressing hsp70 have been reported to decrease infarct size [Plumier et al., 1995] and extra gene copies have been shown to support cell integrity, viability, and function in tissues such as the heart [Yellon and Latchman, 1992; Iwaki et al., 1993; Udelsman et al., 1993; Welch and Brown, 1993; Williams et al., 1993; Plumier and Currie, 1996; Benjamin and McMillan, 1998].

Most damage incurred by ischemic events is suffered at reperfusion, when blood flow and oxygen supplies resume [Yellon and Latchman, 1992]. It is believed that generation of high concentrations of oxygen radicals during this period are responsible for extensive cellular damage. In many cases the endogenous synthesis of stress proteins, induced primarily at reperfusion, is too late to prevent significant damage. In an embryonic chick heart model, EM field-induced protection is also conferred when the fields are activated *after* hypoxic conditions have developed, but prior to reperfusion [DiCarlo et al., 1999].

Pretreatment with heat stress has been successfully used with whole animals to improve recovery from ischemia [Donnelly et al., 1992; Currie et al., 1993] and increase resistance of donor hearts to the rigors of cryopreservation and subsequent warming for transplantation [Gowda et al., 1998]. A 100% induction of hsp70 strengthens heart muscle cell resistance to oxidation, ischemia, and hypoxia [Heads et al., 1994; Mestril et al., 1994, 1996; Chong et al., 1998]. During transient myocardial ischemia in vivo, concentrations of endogenous hsp70 have been shown to increase, doubling 1 h after coronary artery occlusion [Loncar et al., 1998]. When increases in stress proteins reach at least 4 to 5× baseline (which may not occur for up to 24 h after the ischemic stress), improved ischemic tolerance (e.g., recovery of cardiac output and left ventricular pressure) in myocardial tissue is noted [Cornelussen et al., 1998]. Unfortunately, this is *outside* the "golden window of opportunity" to protect the mycardium, which is at greatest risk the first 6 h after artery occlusion.

Central to the reduction of risk posed to the heart by all major surgery, and for the treatment of myocardial infarction and heart failure, is the protection of heart muscles against ischemia and hypoxia. Based on data from the model reported here, as well as the increased hsp70 following repeated short EM field exposures [Han et al., 1998], we believe that the induction of hsp70 by EM fields can be an effective and efficient cytoprotective intervention. Investigation with mammalian models is the next step in the development of this technique.

ACKNOWLEDGMENTS

Support for this project was provided by the Robert I. Goodman Fund. We thank Abraham J. Kremer for invaluable editorial assistance.

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