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Application of Magnetic Field–Induced Heat Shock Protein 70 for Presurgical Cytoprotection

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Abstract To develop an alternative to hyperthermia for the induction of hsp70 for presurgical cytoprotection, we investigated the optimal exposure conditions for magnetic field induction of hsp70. Normal human breast cells (HTB124) were exposed to 60-Hz magnetic fields and hsp70 levels were measured following three different exposure conditions: continuous exposure up to 3 h, a single 20-min exposure, and a single 20-min exposure followed by repeated 20-min exposures at different field strengths. In cells exposed continuously for 3 h, hsp70 levels peaked (46%) within 20 min and returned to control levels by 2 h. Following a single 20-min exposure, the return of hsp70 levels to control values extended to more than 3 h. When cells underwent a 20-min exposure followed by repeated 20-min exposures (restimulation) with different field strengths, additional increases in hsp70 levels were induced: 31% at 1 h, 41% at 2 h, and 30% at 3 h. J. Cell. Biochem. 71:577–583, 1998. © 1998 Wiley-Liss, Inc.

Key words: hsp70; translation; heat shock proteins; stress response; restimulation; feedback inhibition

Stress proteins (hsps) are induced in cells in response to such stresses as elevated temperature or anoxia. This evolutionarily conserved mechanism protects cells against a subsequent stress and even lethal stresses. Stress proteins play crucial roles in a wide variety of normal cellular processes [reviewed in Jindal and Young, 1991], and their use in various fields of medicine is increasing [reviewed in Welch, 1992; Jaattela and Wissing, 1992]. Current cardiovascular presurgical protocols apply high temperatures to protect patients against ischemia and reperfusion injury [Currie et al., 1988; Yellon and Latchman, 1992; Donnelly et al., 1992]. Increased myocardial hsp70 expression protects the heart against ischemic injury [Marber et al., 1993; Plumier and Currie, 1996]; whole-body heat stress reduces infarct size and enhances postischemic contractile function [Marber et al., 1995]. Mammalian cells can be protected from cell injury resulting from heat, severe metabolic stress, or simulated ischemia by forced expression of hsp70 [Williams et al., 1992; Heads et al., 1994; Mestril et al., 1994].

Similar protection can be offered by magnetic fields, a much more benign agent. Magnetic field induction of the stress response is an established phenomenon; these low-energy fields induce increased transcript levels for the stress gene HSP70, and elevated levels of hsp70 protein are synthesized [Goodman et al., 1994; Goodman and Blank, 1995; Lin et al., 1997, 1998a]. The induction of the stress response has been shown to protect against subsequent lethal temperatures or anoxia (cross-protection) [DiCarlo et al., 1998; Goodman and Blank, 1998]. An important advantage of magnetic fields for clinical pre-conditioning is that the magnetic stimulus is a small perturbation compared with the effects of high temperatures (heat shock). Magnetic fields evoke the stress response at *14 orders of magnitude lower* energy input than heat shock. Magnetic field stimulation for stress protein induction, there-

Abbreviations used: Hz, Hertz (cycles/s); HS, heat shock; hsps, heat shock proteins; elf, extremely low frequency (below 300Hz); μT, microTesla (1 μT = 10milliGauss)

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fore, offers an attractive alternative to the use of hyperthermia for stress protein induction prior to surgery, with significant advantages for both clinician and patient in its ease and simplicity of application.

To develop a prototype for clinically applicable presurgical cytoprotection by magnetic field induction of hsp70, exposure conditions must be delineated to maximize hsp70 levels. In the experiments reported here, human female breast cells (HTB124) were subjected to three different exposure conditions: continuous up to 3 h, a single short exposure, and repeated short exposures (restimulation) at different field strengths. Recovery (return to control levels) and refractory periods (the time period when cells are no longer responsive to magnetic fields) were established. Restimulation experiments showed that additional increases in hsp70 could be induced by re-exposures to a *different* field strength, either higher or lower.

MATERIALS AND METHODS

Cell Line and Culturing Conditions

HTB124 (aka ATTC100) normal breast cells from a patient with a genetic predisposition to breast cancer were grown in DMEM. Ten percent fetal bovine serum (FBS; Sigma) and penicillin-streptomycin solution (10,000 IU/ml penicillin and 10,000 µg/ml streptomycin) was added to all cultures. The adherent cells were exposed to magnetic fields at subconfluence in Petri dishes. Cells were prepared for each experiment the previous day by aliquoting cells from a single Petri dish, ensuring that control and experimental samples derived from the original batch of cells and that cell concentrations were identical. The medium was not changed again before exposure of cells to the magnetic field.

60-Hz Magnetic Field Exposure System

Magnetic fields were generated by double-wrapped Helmholtz coils (164 turns of 19-gauge copper wire around a 13 × 14-cm Plexiglas form (Electric Research Management, Pittsburgh, PA). Coils were activated *prior* to placing the cells in the coils to avoid exposing cells to transients. Cells were removed from the field *prior* to deactivating the coils. Mu metal boxes (Ammuneal Manufact. Corp., Philadelphia, PA) shield the coils from stray fields and the geomagnetic fields within the incubator. The sinusoidal field was generated by a Wavetek function generator (Wavetek model 21, 11 MHz) connected to a power regulator. The function generator and power regulator were situated outside the incubator (NuAire). Signal parameters were monitored by a calibrated inductive search coil (Electro-Biology, Inc.) with a Hitachi (V-1065, 100 MHz) oscillscope. There are two identical sets of coils, and each can be set for either active (exposure) or zero field (sham-exposed mode). Controls were cells that were sham-exposed (where the direction of current flow creates a zero field). Dishes or flasks containing cells for experimental or sham exposures were set within an area of the coils having a uniform field.

Temperatures were monitored with a thermocouple probe (PhysiTemp, Inc., Hackensack, NJ) attached to the Helmholtz coils throughout all magnetic field exposures (sensitivity ± 0.1°C).

Magnetic Field Exposure Conditions

The effects of magnetic fields on hsp70 levels were examined with three different exposure conditions.

Continuous exposures. Cells were exposed (8 µT 60 Hz), and protein extracts were prepared hourly up to 3 h.

Single exposure limited to 20 min. Cells were exposed (8 µT or 80 µT, 60 Hz) for 20 min only, removed from the field, and protein was extracted at 1, 2, and 3 h *following* the exposure.

Single 20-min exposure followed by repeated 20-min exposures (restimulation). Cells were exposed (8 µT or 80 µT, 60 Hz) for 20 min only, and then *restimulated* for 20 min with either the same or a different field strength at 1, 2, and 3 h following the initial single 20-min exposure.

Each 20-min exposure was followed by an additional 20-min period out of the magnetic field (37°C) prior to protein extraction. In some experiments cells stimulated with magnetic fields and in refraction (no longer responsive to magnetic fields) were heat-shocked for 40 min at 43°C.

Heat Shock

Petri dishes, containing cells, were wrapped in Parafilm and placed in a mu metal box to protect cells from exposure to magnetic fields generated by the heating device in the water bath. Cells were immersed in a water bath at 43°C for 20 min, followed by an additional 20

min at 37°C before protein extraction. In some experiments, cells initially heat-shocked and in refraction (no longer responsive to heat shock) were exposed to magnetic fields.

Preparation of Protein Lysates

Cells were harvested, washed with cold PBS, centrifuged, and rapidly frozen at −70°C. Lysates were prepared from whole cells using a modification of Mosser et al. [1988; Lin et al., 1997]. The frozen pellets were extracted in a buffer containing 20 mM HEPES, pH 7.9, 25% (vol/vol) glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 1.0 mM dithiothreoitol and centrifuged at 14,000 rpm for 20 min. The supernatants were frozen in liquid nitrogen and stored at −70°C. Protein concentrations were determined with a Bio-Rad protein assay kit (Bio-Rad Laboratories).

Western Blot Analyses

Hsp70 protein levels were determined by Western blot analyses [Lin et al., 1997]. Experiments were repeated a minimum of four times, and each sample within an experiment was analyzed "blind" by at least two different members of the technical staff. Protein samples were prepared 20 min *following* each exposure time. Lysates from whole cells were analyzed using the ECL detection system (Amersham). Anti-hsp70 antibody was obtained from StresGen Inc. (Vancouver, Canada; Cat.# SPA 820; Lot 702409). Results were quantified using a PhosphorImager (400A, Molecular Dynamics) and ImageQuant software.

RESULTS

Statistical Analysis of Experimental Variability

Initially we determined the variability in results from experiment to experiment. Protein samples from four separate experiments were analyzed to determine the variability of hsp70 levels in magnetic field–exposed or sham-exposed cells. Exposures were at 8 μT 60 Hz for 20 min (plus an additional 20 min out of the field) (Fig. 1). The average E/C (ratio of experimental to control) was 1.46 with a standard error of the mean (SEM) of 0.013. A paired t-test determined that the difference between experimental and sham-exposed control was significant: $P = .02$.

To characterize the conditions for magnetic field induction of hsp70 for future application in clinical preconditioning protocols, three exposure regimens were compared to measure hsp70 levels.

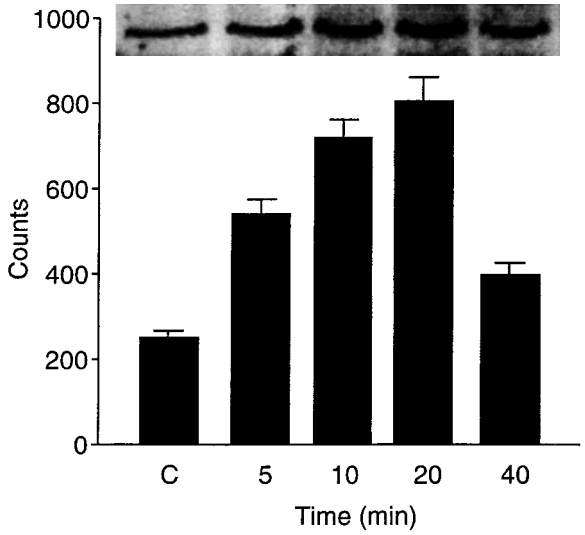

Fig. 1. Variability in hsp70 levels between experiments. Cells were exposed to an 8-μT 60-Hz magnetic field for 40 min. Cell samples were removed for protein analyses at 5-min intervals and maintained out of the field for 35 min before extraction of protein.

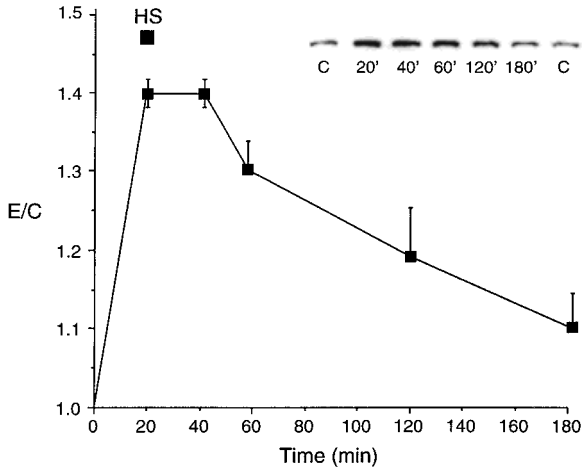

Fig. 2. hsp70 levels in cells exposed to a magnetic field continuously for 3 h. Cells were exposed to a continuous magnetic field, 8 μT 60 Hz. Cell samples were removed for protein extraction at 1-h intervals during exposure.

Continuous Exposure

There was a rapid 46% increase in hsp70 levels within 20 min in cells continuously exposed to magnetic fields and a return to control levels by 2 h (Fig. 2).

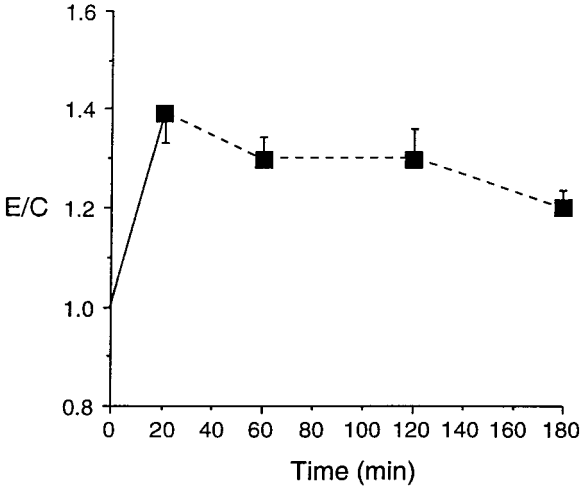

Fig. 3. hsp 70 levels in cells exposed to magnetic fields for only 20 min. Cells were exposed to a single 20-min exposure, 8 μT 60 Hz, and then removed from the field for 3 h. Cell samples were removed for protein extraction at 1-h intervals.

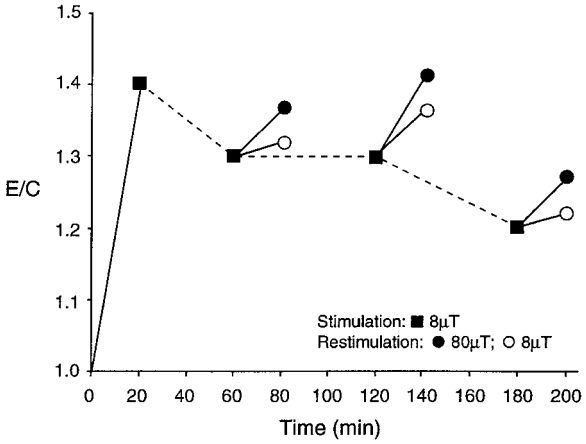

Fig. 4. Restimulation of hsp70 levels: 8 μT restimulated by 8 μT and 80 μT. Cells were exposed for 20 min to an 8-μT 60-Hz magnetic field and then restimulated with the same field strength (8 μT) for 20 min, or a higher field strength (80 μT) for 20 min at 1, 2, and 3 h following the initial 20-min exposure.

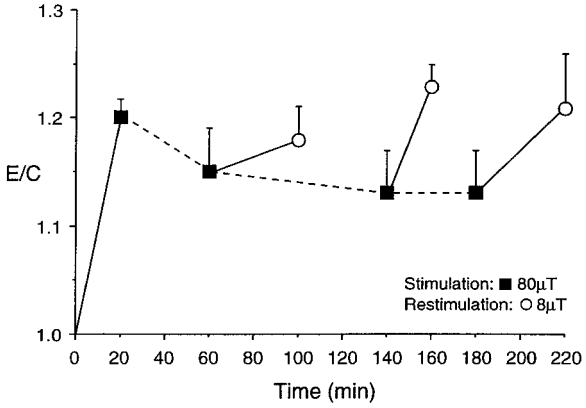

Fig. 5. Restimulation of hsp70 levels: 80 μT restimulated by 80 μT and 8 μT. Restimulation: Cells exposed for 20 min to an 80-μT 60-Hz magnetic field and then restimulated with the same (80 μT) or a lower field strength (8 μT) at 1, 2, and 3 h after the initial exposure. Only a different field strength could restimulate these cells.

A Single 20-Minute Exposure

In cells stimulated by magnetic fields for a single 20-min exposure, the hsp70 levels peaked at 20 min and remained elevated for more than 3 h (Fig. 3).

A Single 20-Minute Exposure Followed by Repeated Exposures of 20-Minutes Each (Restimulation)

Cells exposed to magnetic fields for 20 min and then *re-stimulated* by magnetic fields of different amplitudes had a significant increase in hsp70 levels when the second exposure (re-exposure) was with a field strength *different* from the initial field strength: 31% at 1 h, 41% at 2 h, and 30% at 3 h (Fig. 4). Cells stimulated initially with 80 μT and then restimulated with 8 μT had a small but significant additional increase in hsp70 levels. No restimulation was achieved with the same (80 μT) field strength (Fig. 5).

Cells in a refractory state, initially stimulated by magnetic fields, could not be restimulated by heat shock. Cells in the refractory period, initially stimulated by heat shock, could not be restimulated by magnetic fields (data not shown).

DISCUSSION

Magnetic Field–Induced Stress Response and Cytoprotection

The stress response, an important homeostatic mechanism, enables animal, plant, and bacterial cells to survive a variety of environmental stresses, including heat shock, amino acid analogs, heavy metal ions, and oxidative stress [reviewed in Morimoto et al., 1994]. The cellular stress response is also induced by magnetic fields [Goodman et al., 1994; Goodman and Blank, 1995; Lin et al., 1997, 1998a,b]. The proteins synthesized in thermally conditioned cells protects them from the adverse effects of both initial and subsequent thermal stresses; i.e., the cells become thermotolerant [Gerner and Schneider, 1975; reviewed in Welch, 1992]. The stress response proteins induced by magnetic fields also provide cytoprotection from

both lethal temperatures [Goodman and Blank, 1998] and anoxia [DiCarlo et al., 1998]. The development of thermotolerance and cytoprotection is mediated by hsp70, which functions as an inhibitor of heat shock factor (HSF) [Morimoto et al., 1994].

Cytoprotection as mediated by hsp70 molecular chaperones has been shown to have a role in guarding against stress-related effects associated with aging [reviewed in Tatar et al., 1997]. In thermally conditioned *Drosophila melanogaster* and *C. elegans*, greater longevity resulted when adults were mildly heated and then returned to normal growth temperatures. A brief mild heat shock induced a low level of hsp70 that brought about long-term improvement in survival. The transient expression of molecular chaperones may increase survival through their ability to renature, assemble, and disassemble proteins, as well as interact with other stress-response mechanisms. Transient levels of hsp70 may also repair and restore cellular functions that would otherwise accelerate senescence, and hsp70 may even persist in some specific tissues over the period of increased survival.

Differences Between Magnetic Field Activation and Heat Activation

The many similarities between magnetic field–activated and heat-activated stress response, i.e., increased activation of HSF and HSE binding [Lin et al., 1997], increased HSP70 expression [Goodman et al., 1994; Lin et al., 1997, 1998a], and magnetic field–induced cytoprotection similar to thermotolerance [Goodman and Blank, 1998; DiCarlo et al., 1998], provide convincing evidence that magnetic fields induce the stress response. However, there are important differences:

- Magnetic fields evoke the stress response at *14 orders of magnitude lower* energy input than heat shock.
- Magnetic field-induced stress does not inhibit the synthesis of other proteins in the cell.
- The basal region in the HSP70 promoter responsive to magnetic fields maps to a different domain, −160 to −230, with an HSE at −195 [Lin et al., 1998a; Goodman and Blank, 1998] than that of heat shock, which is at −68 to −106, with an HSE at −100 [Williams and Morimoto, 1990].
- Myc-binding sites in the HSP70 promoter (−160 and −230) are essential in the magnetic field induction of HSP70 [Lin et al., 1998a], but not for HSP70 induction by heat shock.
- HSP70 expression by magnetic fields *requires* Myc protein [Lin et al., 1998a].
- Magnetic fields induce increased DNA-binding activity of AP-1 (fos/jun protein) [Lin et al., 1998b]; heat shock does not.

The restimulation experiments reveal an unusual aspect of initiation of the stress response with magnetic fields. Cells stimulated with magnetic fields, and then re-stimulated 1, 2, and 3 h later, respond to a *different* magnetic field strength with a further increase in hsp70 levels, i.e., **cells respond to the different field strength as if it were a new stimulus.** This observation is similar to earlier studies in which we examined the effect of magnetic fields on *myc* transcript levels during exposures and re-exposures [Lin et al., 1996]. Both studies show that the inhibitory mechanisms leading to a refractory period can differentiate between exposures that differ in field strength by an order of magnitude.

The significant differences between magnetic field-activated and heat-activated stress response raise questions regarding the signaling pathways activated by the two stimuli. A circuit diagram of possible signaling pathways implicated in both thermal stress and magnetic field-induced stress is presented in Figure 6. Based on magnetic field-induced binding-activation of *both* HSF and AP-1, cellular changes induced by magnetic fields may well result from activa-

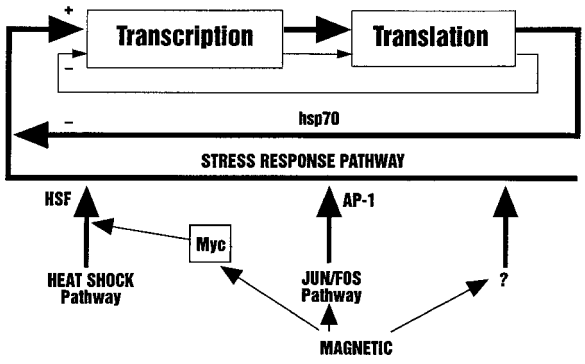

Fig. 6. Circuit diagram Magnetic fields may interact with the two signaling pathways: 1) the stress-activated protein kinase (SAPK) cascade terminating in the phosphorylation and activation of the AP-1 by jun N-terminal kinase (JNK) and p38, and 2) the stress-activated pathway that induces nuclear translocation of the HSF monomer, HSF trimerization, and HSE-binding. In this model control of biosynthesis is effected by feedback loops in cells stimulated and restimulated by magnetic fields or heat shock.

tion of *both* the JUN/FOS pathway (stress-activated protein kinase cascade terminating in the phosphorylation and activation of the AP-1 by jun N-terminal kinase and p38), as well as the HEAT SHOCK pathway (which induces nuclear translocation of the HSF monomer, HSF trimerization, and HSE binding). Alternatively, one pathway may be activated and may intersect and converge with the other pathway [Hopkin, 1997]. The role of Myc protein as an essential transcription factor in the magnetic field mechanism is important in this process, but the details of this interaction remain to be determined.

The conventional stress-activated signal transduction pathways may not be the only mechanism for extracellular signaling to the nucleus. The demonstration that magnetic fields stimulate transcription in cell-free preparations [Goodman et al., 1993; Tuinstra et al., 1997] implies that membrane interaction is not an essential element in transmitting the signal to the nucleus. Since magnetic fields can penetrate cells and are not limited to interactions with the membrane, a direct effect of magnetic fields on the DNA has been proposed [Blank and Goodman, 1997] through interaction with the conducting electrons in the stacked bases of the DNA [Dandliker et al., 1997]. The electron flow within the stacked bases of DNA may be of sufficient magnitude to interact with the relatively weak magnetic fields that stimulate transcription. The data presented show that cells respond to different field strengths as if they were new stimuli and suggest that different segments of DNA may be activated.

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