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Chronic Electromagnetic Field Exposure Decreases HSP70 Levels and Lowers Cytoprotection

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Abstract Electromagnetic field (EMF) exposures have been shown to induce heat shock proteins (HSPs), which help to maintain the conformation of cellular proteins during periods of stress. We have previously reported that short-term exposure of chick embryos to either 60 Hz (extremely low frequency: ELF), or radio-frequency (RF: 915 MHz) EMFs induce protection against hypoxia. Experiments presented in the current report are based on a study in which long-term (4 days), continuous exposure to ELF-EMFs *decreased* protection against ultraviolet radiation. Based on this result, it was hypothesized that de-protection against hypoxia should also occur following long-term, continuous, or daily, repeated exposures to EMFs. To test this hypothesis, chick embryos were exposed to ELF-EMFs (8 μT) continuously for 4 days, or to ELF or RF (3.5 mW incident power)-EMFs repeated daily (20, 30, or 60 min once or twice daily for 4 days). Several of the exposure protocols yielded embryos that had statistically significant decreases in protection against hypoxic stress (continuous and 30 or 60 min ELF twice daily; or 30 or 60 min once daily RF). This is consistent with our finding that following 4 days of ELF-EMF exposure, HSP70 levels decline by 27% as compared to controls. In addition, the superposition of ELF-EM noise, previously shown to minimize ELF-EMF induced hypoxia protection, inhibited hypoxia de-protection caused by long term, continuous ELF or daily, repeated RF exposures. This EMF-induced decrease in HSP70 levels and resulting decline in cytoprotection suggests a mechanism by which daily exposure (such as might be experienced by mobile phone users) could enhance the probability of cancer and other diseases. J. Cell. Biochem. 84: 447–454, 2002.

Key words: electromagnetic fields; non-ionizing radiation; radio-frequency; microwave; HSP70; continuous; repeated; noise; stress proteins; cancer; mobile phones

We have recently reported that extremely low frequency (ELF) electromagnetic field (EMF) exposure of chick embryos induces protection during subsequent hypoxia [Di Carlo et al., 1999a] or ultraviolet (UV) light irradiation [Di Carlo et al., 1999b], suggesting that EMF exposures may be beneficial in short doses. In part, the mechanism for this cytoprotection is believed to be activation of the heat shock response pathway. Activation of this stress response by EMF, specifically, enhanced heat shock protein 70 (HSP70) levels, was first shown in cell cultures [Goodman et al., 1994; Lin et al., 1997; Han et al., 1998]. These findings have since been strengthened by reports in a number of different experimental models and exposure protocols, indicating that EMF exposures can enhance HSP70 levels either alone, or in combination with other stressors [Cairo et al., 1998; Daniells et al., 1998; Pipkin et al., 1999; Tsurita et al., 1999; Chow and Tung, 2000; Junkersdorf et al., 2000]. In a review of EMFs and the HSP response by Goodman and Blank [1998], they pointed out that multiple stresses (such as hyperthermia and EMFs) can act through a common pathway mediated by HSP70, thus the finding that EMFs can confer cytoprotection is not unusual. In addition, previous reports from this laboratory of EMF-induced hypoxia protection are consistent with results from Albertini et al. [1999], which indicate that EMF exposures can minimize in vivo myocardial damage in rats following coronary artery occlusion, and Grant et al. [1994], who reported that EMF exposure improved outcomes following focal cerebral ischemia in rabbits.

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We hypothesized that, in a manner similar to that seen for chronic exposure to retinoids [Tosi et al., 1997], hypoxia [Oehler et al., 2000], or heavy metals [Somji et al., 1999a, 1999b; Croute et al., 2000], which have been shown to lower heat shock protein levels, long-term or repeated exposure to EMFs might also "exhaust" cellular protective mechanisms by decreasing HSP70 levels. This concept was previously studied by Lin et al. [1996], who showed feedback effects of HSP70 in a study comparing continuous and intermittent EMF exposures. We further hypothesized that over time, the downregulation of the mechanisms responsible for this protection would lead to an even greater susceptibility to a secondary insult, and this decreased protection would correlate with HSP70 levels. Therefore, experiments were designed in which chick embryos were either continuously or repeatedly exposed to ELF- or radio frequency (RF)-EMFs to assay for HSP70 induction or survival following hypoxia/re-oxygenation insult.

MATERIALS AND METHODS

Embryos

Fertilized White Leghorn eggs (Truslow Farms, Chestertown, MD) were placed into a refrigerator (10°C, ambient magnetic fields below 0.5 μT) for up to 48 h from time of receipt. During incubations, embryos were held in water-jacketed incubators (VWR) (37.8 ± 0.1°C, > 55% humidity) for 4 days. Developmental stages ranged from 22 to 25 [Hamburger and Hamilton, 1951]. Eggs were randomly distributed in cartons among the incubators and periodically moved to eliminate any incubator-specific effect.

ELF-EMF Exposures

The ability of 60 Hz ELF-EMFs to induce a response in the chick embryo were tested for short-term (protective: one-time, 30 min) or long-term (de-protective: 4 days continuous, or 20, 30, or 60 min once or twice daily repeated) exposure. The 60 Hz signals were generated with a 15-MHz function/arbitrary waveform generator (Hewlett-Packard model 33120A) and a 100 W P.A. amplifier (Radio Shack). Fields were produced by passing current through paired coils arranged in the Helmholtz configuration, wound and connected as described previously with slight modification [Berman et al., 1990]. A coil diameter of 15 inches provided a region of nearly uniform magnetic field (± 5%). Up to 24 embryos could be exposed at one time. Each of six coils was housed within a water-jacketed incubator maintained at 37.8°C. Ambient magnetic fields in the incubators were very low (below 0.5 μT) at all egg positions. Magnetic fields were measured using a 60 Hz-calibrated dosimeter (Model IDR-109; Integrity Design and Research Corporation). Random ELF-EMF noise (band width 30–90 Hz, 8 μT) was produced using a random noise generator built at Catholic University, incorporated into a 35-W audio amplifier and attached to the Helmholtz coils as described above. Paired Helmholtz coils were wired such that one of the pair could be configured to produce a canceled field while the other coil of the pair produced a 60 Hz field. Thus, control (sham-exposed) embryos received no field exposure, but did experience all other environmental conditions (e.g., generation of heat or vibration) that might be produced by an activated coil.

All ELF-EMF exposures were done at 37.8°C. For HSP70 induction and protection assays, embryos were exposed for 30 min, once on incubation day 4. For hypoxia de-protection experiments, embryos were exposed continuously (4 days), or 20, 30, or 60 min, once or twice daily. Exposures were done at midnight (for once daily exposures) or midnight and noon (for twice daily exposure). All hypoxia challenge experiments were initiated on the morning of incubation day 4.

RF-EMF Exposures

The ability of 915 MHz, 3.5 W incident power RF-EMFs to induce a response in the chick embryo was tested for short-term (protective: one time, 30 min) or repeated daily (de-protective: 30 or 60 min once daily) exposures. Microwave exposures were done with a Crawford cell (Model CC110; Instruments for Industry, Farmingdale, NY) in an incubator chamber maintained at 37.5°C. The Crawford cell, was vertically-mounted on a rotary table, allowing access to two sample chambers located on either side of the center conductor, with simultaneous exposure of eight embryos (in shells). A signal generator (Model 8567B with RF plug-in 83522A; Hewlett Packard) was used as the microwave signal source followed by a 10 W solid-state traveling wave tube amplifier (Model 10W1000; Amplifier Research, Souderton, PA). A double stub tuner was used to match the

impedance of the loaded Crawford cell for optimum power delivery. The specific absorption rate (SAR) calculated from the 3.5 W incident power exposure was approximately 1.7 W/Kg. Control embryos were placed outside the Crawford cell. Thermocouple readings taken at the site of the embryo during the 30 or 60 min RF-EMF exposures indicated that the temperature increase was less than 0.6 or 1.2°C, respectively (to below 39.0°C final temperature) and thus is insufficient to activate the thermally-induced heat shock protein pathway. It has been demonstrated that chicken cells require temperature increases of at least 4°C to activate the heat shock protein response [Schlesinger, 1985].

HSP70 Assay

Positive control embryos for the HSP70 induction assay were heated by placing eggs (with embryos) into plastic bags and submerging them in a 43°C water bath (±0.2°C). Embryos were maintained for 60 min, at which time they were removed from the bath and allowed to cool in ambient air (21°C) for 5 min before being placed into a 37.8°C incubator. After 3 h from the start of heating, embryos were assayed for HSP70 levels.

After heating, or ELF-EMF exposure (30 min or 4 days), embryos (4–5/sample) were removed from the eggs by cutting blood vessels near the head and tail region and plucking the embryo out. Other chick embryo samples were heated at 39 or 41°C for 30 min with a 2.5 h wait before sampling to mimic the heating which might possibly occur during an RF-EMF exposure. Embryos were placed into 1 ml of ice-cold chick Ringers solution (0.12 M NaCl, 0.005 M KCl, 0.01 M $CaCl_2$), and disrupted by sonication (Model 60 Sonic Dismembranator; Fisher Scientific), followed by centrifugation at 12,000*g* (4°C). Supernatants were collected and HSP70 levels were measured by Western blotting. Ten micrograms of total protein for each sample were run on an SDS–polyacrylamide electrophoresis gel and transferred to a nitrocellulose membrane (Amersham, Piscataway, NJ) which was blocked overnight with 5% non-fat milk. First antibody (rabbit anti-chicken HSP70 [Kawazoe et al., 1999]; diluted 1:10,000), and second antibody (horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (Cappel, Durham, NC; diluted 1:20,000)) were used to discern protein bands. HSP70-positive bands were detected using chemiluminescence reagents (ECL plus, Amersham, Piscataway, NJ) and exposure of X-ray film (Hyperfilm ECL; Amersham). Films were photographed, and band densities (IOD) were quantified using Gel Pro image software (Media Cybernetics, Silver Spring, MD). After heating at 39°C, no increase in HSP70 levels was noted (data not shown). Slight elevation of HSP70 was observed at 41°C. Therefore, the temperature rise measured during our RF-EMF exposure (up to 39°C) would not be expected to activate the thermal, heat shock protein pathway.

Embryo Preparation-Hypoxia Protection Study

Following RF or ELF exposure, eggs were windowed to allow for observation of the embryos and facilitate hypoxia. To achieve blinding, embryos were coded by one individual and the code was entered into a computer program designed to keep the observer blinded during evaluations. Hypoxic conditions were initiated as described previously [Di Carlo et al., 1999a]. Briefly, coded embryos were placed into airtight bags such that each treatment condition was equally represented in each bag. Air was evacuated using gentle suction, and bags were then filled with argon and returned to the incubators (37.8°C). At 30-min intervals, embryos were evaluated by observing heart beat through the bag. Mortality data (heart beating or stopped) were entered into the computer program that, without identifying which eggs were control or exposed, provided the researcher with percent survival for the embryos in each bag. For the 30 min, one-time (protective) ELF- and RF-EMF exposures only, bags were opened for reoxygenation when control embryo hearts were reduced to 15–45% still beating. For the other EMF experiments (both ELF and RF), in which downregulation of protection was hypothesized, bags were opened when EMF-exposed embryo hearts were reduced to 15–45% still beating. Only experiments in which final survival was between 15–45% in the targeted embryos were considered in final data. This targeted percent survival range was chosen because survival below 15% indicated the timing of the hypoxic insult was too long, and survival above 45% suggested that hypoxia was not administered long enough to observe differential protection between control and exposed embryos. Final observations (reported here) were made after 30 min recovery in 37.8°C.

Statistics

Standard error of the mean (SEM) was determined for all data points. The P values and statistical significance of tabular data were determined with Fisher's exact test. The P values for the HSP70 induction results (Fig. 1) were determined using the two-tailed, paired Student's t-test. All statistics were calculated using Instat© (1993) (Version 2.04; GraphPad Software, San Diego, CA).

RESULTS

ELF-EMF exposure (60 Hz, 8 μT) induced changes in HSP70 levels that were quantified by Western blotting. Figure 1 shows a representative blot of chick embryo lysates taken after heating, or short- or long-term EMF treatment. A total of 14 sets of samples were run on six different days to generate data presented. Control embryos, not treated with EMF or heat, had detectable levels of HSP70 (average band integrated optical density (IOD) = 120). Because they are an embryonic system involved in rapid development, the finding of HSP70 in otherwise non-stressed controls is not unusual, and has been reported in earlier studies [Kawazoe et al., 1999]. Consistently measurable increases (30–70% above control) in HSP70 levels were noted at 3 h from the start of the 30 min exposure (EMF 3 h). Lysates from those embryos which had been exposed to ELF-EMFs for 4 days prior to assaying had significantly lower levels of HSP70 (average band IOD = 84, $P < 0.02$) as compared to controls. Embryos heated at 43°C with no EMF-treatment showed increases in HSP70 levels of 250–1000% over controls. Other embryos were also heated at 39 and 41°C for 30 min with a 2.5-h wait before assaying to confirm the temperature at which the thermally-induced heat shock protein response would be initiated (data not shown). No increase in HSP70 was noted at the 39°C incubation. Relative to the 43°C heating, smaller HSP increases (85% over controls) were noted with the 41°C incubation.

Table I gives data for exposure of embryos to ELF-EMFs, either short-term (one time on day 4), continuously (4 days) or repeated, daily (20, 30, or 60 min over 4 days) prior to hypoxia administered on incubation day 4. As has been reported previously [Di Carlo et al., 1999a], single exposure to ELF-EMFs yields a statistically-significant increase in hypoxia protection ($P < 0.01$). However, in those embryos continuously exposed to ELF-EMFs, a significant *decrease* in protection was noted as compared to survival values for non-exposed controls ($P < 0.01$). This de-protective effect was mitigated by super-position of an equivalent strength ELF-EM noise field ($P = 0.548$).

Having shown that continuous exposure decreased protection, we next sought to determine if repeated exposure would lead to decreased protection. Table I data show that continuous embryo exposure is not needed to achieve significant de-protection following hypoxia. Significant de-protection was also achieved by exposing embryos to ELF-EMF for 30 min ($P < 0.05$) or 60 min, ($P < 0.01$) twice daily for 4 days prior to hypoxia. Thirty min exposures, once daily ($P = 0.068$) and 20 min exposures, twice daily, however, were ineffective ($P = 0.548$) at altering hypoxia protection.

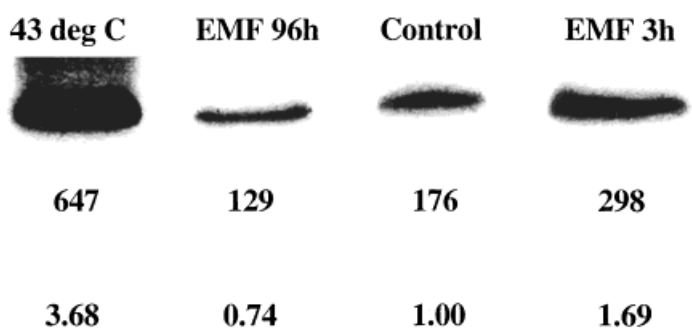

Fig. 1. HSP70 levels in 4 day-old chick embryos exposed to ELF-EMFs and heat shock. A representative Western blot is shown. **Lane 1**: HSP70 in embryos exposed to 43°C for 1 h followed by a 2-h wait. **Lane 2**: HSP70 in embryos chronically exposed to 60 Hz, 8 μT EMF for 4 days and assayed immediately. **Lane 3**: HSP70 in non EMF-exposed embryos. **Lane 4**: HSP70 in emryos exposed once to 60 Hz, 8 μT EMF (1 h) and assayed 2 h later (3 h total).

TABLE I. Continuous or Repeated Exposures to ELF-EMFs Decrease Protection Against Hypoxia/Re-Oxygenation Stress

ELF exposure timing (60 Hz, 8 μT)	Control % survival ± SEM (# embryos)	ELF-EM % survival ± SEM (# embryos)	N (reps)	*P* value*
30 min (one time only, Day 4)†	24.1 ± 4.1 (87)	44.9 ± 6.0 (78)	9	< 0.01
Continuous (4 days)	66.2 ± 6.3 (65)	40.6 ± 3.4 (64)	8	< 0.01
Continuous + 8 μT ELF noise	66.2 ± 6.3 (65)	72.0 ± 6.4 (50)	8	0.548
60 min twice daily (4 days total)	53.0 ± 5.2 (168)	38.1 ± 3.0 (160)	16	< 0.01
30 min twice daily (4 days total)	50.9 ± 6.7 (57)	26.5 ± 6.7 (49)	7	< 0.05
20 min twice daily (4 days total)	34.8 ± 7.0 (69)	35.2 ± 5.6 (71)	8	1.0
30 min once daily (4 days total)	47.5 ± 6.7 (122)	35.0 ± 2.8 (117)	13	0.068

*P values calculated by Fisher's exact test.
†Upregulation positive control.

Given data which indicated that continuous or repeated exposure to ELF-EMFs decreased hypoxia protection, we next tested whether repeated daily RF-EMF exposures decreased protection. Our laboratory has found that RF-EMFs cause many of the same biochemical changes within tissues as ELF-EMFs [Penafiel et al., 1997]. For example, embryos exposed one-time only to RF-EMFs (30 min on day 4, 3.5 W incident power) were found to have significantly higher survival following hypoxia than controls (data not shown). This is similar to results that were seen with one-time ELF-EMF exposures (Table I). Table II shows results from studies in which embryos were exposed intermittently (30 or 60 min once daily over four incubation days) to RF-EMFs (3.5 W incident power, ~SAR of 1.7 W/Kg). Continuous exposures were not done for RF-EMF due to the possibility of significant heating effects. Both daily RF-EMF exposures tested (30 or 60 min once daily) yielded decreases in hypoxia protection ($P < 0.05$ and < 0.01, respectively). The de-protection noted following the 30 min once daily RF-EMF exposures was mitigated by super-position of an 8 μT ELF-EM noise field ($P = 0.86$).

DISCUSSION

Our results demonstrate that duration and timing of exposure will determine whether EMF-induced biological effects are beneficial or adverse, indicating that although short, single exposures to EMFs can be protective during hypoxia, continuous or repeated, daily exposures can lead to de-protection. This finding is consistent with our previous work, showing that long-term exposure to EMFs decreased protection against UV radiation stress [Di Carlo et al., 1999b]. In that study, short-term, single EMF exposures gave a 60% increase in protection, yet continuous exposures rendered embryos more susceptible to UV damage than non-EMF-exposed controls (30% decrease in survival). Typical environmental exposure to ELF-EMFs (in the absence of any electrical equipment) are on the order of less than 0.2 μT (as compared to the 8 μT fields used in this study), however, occupational exposures may exceed 10 μT. For RF exposures, by law, cell phones must have an SAR of less than 1.6. Although the RF-EMFs used in the present study exceed this level, lower SAR exposures have not yet been tested to determine if they can elicit the same alterations in hypoxia protection.

We have also demonstrated that chronic exposure to an ELF-EM field can lead to decreased levels of HSP70. This finding is not unexpected, however, as a survey of the literature finds a number of studies in which HSP

TABLE II. Continuous or Repeated Exposures to RF-EMFs Decrease Protection Against Hypoxia/Re-Oxygenation Stress

RF exposure timing (915 MHz, 3.5 W)	Control % survival ± SEM (# embryos)	RE-EM % survival ± SEM (# embryos)	N (reps)	*P* value*
60 min once daily (4 days total)	51.7 ± 9.1 (58)	26.4 ± 3.6 (54)	8	< 0.01
30 min once daily (4 days total)	45.5 ± 4.4 (66)	27.3 ± 3.1 (66)	9	< 0.05
30 min once daily + 20 μT ELF Noise	28.8 ± 4.4 (73)	27.4 ± 2.4 (77)	11	0.86

*P values calculated by Fisher's exact test.

levels were found to be down-regulated in response to chronic stress. For example, Tosi et al. [1997] demonstrated that prolonged treatment of human leukemia cells with retinoic acid reduced HSP70 levels, and a study of endothelial cells [Oehler et al., 2000] showed that cells grown for 20 h in a hypoxic environment had HSP70 levels that were reduced as compared to cells grown normally. There are also reports that chronic (1 month) exposure of human lung cells to cadmium can decrease HSP70 induction [Croute et al., 2000]. This finding was strengthened by the study undertaken by Somji et al. [1999a, 2000], who showed that when human proximal tubule cells were chronically (14 days) exposed to $CdCl_2$, HSP60 and HSP27 protein levels were decreased as compared to controls. Finally, in a study on lice continuously maintained on soil that contained known toxins [Kohler et al., 1999], researchers saw that, despite continuous exposure to several toxins, HSP70 levels decreased (after peaking at 24 h) to levels below controls. All of these findings of decreased HSP70 levels following prolonged exposure to a stressor are consistent with our result of decreased hypoxia protection following prolonged or repeated exposure to EMFs.

Results of the present study also indicate that the superposition of ELF noise inhibits RF- and ELF-EMF induced de-protection. This finding is consistent with previous reports from this laboratory [Farrell et al., 1998; Mullins et al., 1998; Di Carlo et al., 1999a] and from others [Lin and Goodman, 1995; Martin and Moses, 1995; Raskmark and Kwee, 1998] that indicate that ELF-EM noise can minimize EMF-induced biological effects. The finding that ELF-EM noise can block de-protection from repeated-daily RF exposure also supports the claim that this RF exposure is not thermally activating the stress protein response system of the embryos since it is our hypothesis that electromagnetic noise cannot block a true thermal effect.

The results presented here also support the hypothesis that cells respond similarly to RF and ELF-EMF exposures, in that hypoxia de-protection was seen for both types of exposure. In addition to the difference in the frequencies of ELF and RF-EMF (in the Hz range for ELF vs. the MHZ range for RF), RF-EMFs have lower penetration than ELF-EMFs into biological tissues, but RF-EMFs have the potential to induce much higher induced electric field voltages in biological tissues. The idea that cells might respond similarly to these two types of EMF exposure was discussed by Penafiel et al. [1997], who showed that both RF and ELF-EMF exposures altered ODC activity similarly in EMF-exposed murine fibroblast (L929) cells.

The present finding of decreased HSP70 levels and hypoxia protection following chronic or repeated daily exposure to EMFs has several implications with regards to human health. Cells are constantly under attack from oxygen radicals generated by routine metabolism. These reactive oxygen species (ROS) can be very damaging to cells, especially to DNA [Pollycove and Feinendegen, 1999]. Of the millions of ROS generated per cell per day, most are neutralized by anti-oxidant molecules such as HSP70 [Musch et al., 1998; Uruyama et al., 1998] and melatonin [Pierrefiche et al., 1993; Oxenkrug et al., 2001], and are thus prevented from causing damage. The ROS that remain, however, can oxidatively modify and disrupt DNA structure. This damage is repaired by enzymes that remove or replace the damaged bases. Because HSP70 induced by short EMF is involved in enhanced DNA repair in bacteria, presumably via protection of repair enzymes [Chow and Tung, 2000], HSP70 levels may indirectly affect DNA structure. If significant DNA errors are left unrepaired by the enzymes, the immune system will normally eliminate those cells. The HSP70 molecule is also involved in this removal step because abnormal cells often express HSP70 on their surface that trigger NK cells to destroy the cell [Multhoff et al., 1997]. There are, however, some cells which elude these prevention and repair mechanisms. As mutations accumulate, they can lead to accelerated aging and the possibility of cancer formation. Thus, if continuous or repeated exposure to external stimuli (such as EMFs) lowers the level of cellular HSP70 (as has been shown in the present report), this could lead to an accumulation of damage by interfering with the body's prevention, repair, and removal processes.

In conclusion, our results indicate that long-term continuous, or daily repeated EMF exposure can decrease HSP70 levels and thus, reduce protection against a subsequent stressor. Also, because HSP70 is involved at all stages of the body's damage response system, decreasing its levels within the cell can lead to an accumulation of DNA errors. These findings have important implications with regards to

potential dangers from prolonged and repeated exposure to non-ionizing radiation (e.g., mobile phone radiation). Whereas daily 20-min exposures did not cause a measurable biological effect, exposures of 30 min or more on a daily basis may lead to a down-regulation of innate stress protective mechanisms. Our repeated EMF exposure protocols, which reflect both daily mobile phone usage and occupational exposure, suggest a mechanism to explain epidemiological studies which find enhanced probability of cancer [Hardell and Mild, 2001] and Alzheimer's disease [Sobel et al., 1996].

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