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Microwave Exposure Induces Hsp70 and Confers Protection Against Hypoxia in Chick Embryos

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Abstract To determine if microwave exposure could elicit a biological effect in the absence of thermal stress, studies were designed in which chick embryos were exposed to athermal microwave radiation (915 MHz) to look for induction of Hsp70, a protein produced during times of cellular stress that aids in the protection of cellular components. Levels of Hsp70 were found to increase within 2 h, with maximum expression (~30% higher than controls) typically occurring by 3 h from the start of exposure. Other embryos were exposed to microwave radiation prior to being subjected to hypoxic stress, and were found to have significantly higher survival ($P < 0.05$) following re-oxygenation than non-exposed controls. The results of these studies indicate that not only can athermal microwave exposures activate the stress protein response pathway; they can also enhance survivability following exposure to a subsequent, potentially lethal stress. From a public health standpoint, it is important that more studies be performed to determine if repeated exposures, a condition likely to be found in cell phone use, are still beneficial. J. Cell. Biochem. 86: 490–496, 2002.

Key words: electromagnetic fields; athermal microwave radiation; stress proteins

A novel biomarker, the inducible heat shock protein (Hsp70), has been reported to be useful for the investigation of microwave-induced cellular stress [Daniells et al., 1998]. Since the inducible form of Hsp70 is not usually detectable in cells under normal, non-stressful conditions, it serves as a highly sensitive indicator of environmental stress. Increased transcription of the heat shock family of proteins can be activated by a number of stimuli such as heat, toxic chemicals, reactive oxygen species, and other stressors [Kiang and Tsokas, 1998]. Because heat shock proteins can be induced by stimuli other than heat, the name is a bit of a misnomer. Thus, to avoid confusion, Hsps are often referred to as stress proteins. Stress proteins, specifically Hsp70, are primarily responsible for maintaining the conformation of other proteins during times of cellular challenge. The Hsp70 molecule is remarkable, in that its induction by a mild stress can protect the cell against damage from a subsequent, potentially lethal stress [Sharp et al., 1999]. This pre-conditioning phenomenon has been shown to protect against a variety of stressors including heat [Landry et al., 1982], ultraviolet light [Trautinger et al., 1995], and ischemia [Mestril and Dillman, 1995].

In addition to the stimuli listed above, extremely low frequency electromagnetic (ELF-EM) fields at frequencies of 50 and 60 Hz have been shown to enhance stress protein levels and alter metabolism in a number of models including cell cultures [Han et al., 1998; Pipkin et al., 1999; Tsurita et al., 1999], *Caenorhabditis elegans* [Junkersdorf et al., 2000], *Drosophila melanogaster* [Michel and Gutze, 1999], and *Escherichia coli* [Chow and Tung, 2000]. Studies in our

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laboratory in the chick embryo model have shown that 60-Hz magnetic field exposures, which are sufficient to induce Hsps, can precondition against damage from subsequent stressors such as hypoxia [Di Carlo et al., 1999a] and ultraviolet light exposure [Di Carlo et al., 1999b]. Embryos pre-treated with microtesla (μT) levels of 60-Hz magnetic fields prior to exposure to a secondary insult showed significantly higher survival rates than non-EM field-exposed controls. The finding that EM field exposures can induce oxidative stress protection is strengthened by work from other researchers, who found that ELF-EM field exposures could also confer protection against heart cell hypoxia damage [Carmody et al., 2000], lethal hyperthermia in *Sciara coprophila* eggs [Goodman and Blank, 1998], cardiac ischemia in rats [Albertini et al., 1999], and cerebral ischemia in rabbits [Grant et al., 1994].

Recently, de Pomerai et al. [2000] reported that athermal radio frequency (RF or microwave) exposures could activate stress protein production. These *C. elegans* studies indicated that Hsp70 levels could be increased by exposure of the nematodes to microwaves (750 MHz) at power levels, which caused less than a 0.5°C increase in body temperature. However, Cleary et al. [1997] saw no increase in Hsps following exposure of HeLa and CHO cells to athermal microwaves (2.45 GHz). It is important to determine if athermal microwave effects exists because of health concerns related to exposure to RF (microwave) radiation from cellular telephones. Current exposure limits are based upon thermal effects and assume that there are no athermal effects. Although there have been several reports of biological effects resulting from athermal microwave exposures [Byus et al., 1984; Lai et al., 1992; Zotti-Martelli et al., 2000], controversy still exists over their ability to induce a biological effect.

To further investigate the possibility of athermal microwave-induced biological effects, we designed a study in which chick embryos were exposed to RF-EM fields (3.5 or 5.0 W incident power), where the temperature rise was kept below 1.5°C for the higher power RF exposure. This temperature rise is below that needed to thermally induce a stress protein response in the chick embryo [Schlesinger, 1985]. Induction of Hsp70 and oxidative stress protection were the markers chosen to demonstrate any microwave-induced biological effect.

MATERIALS AND METHODS

Embryo Preparation

Fertilized White Leghorn eggs (Truslow Farms, Chestertown, MD) were held at 10°C and used within 48 h of receipt. The holding refrigerator was monitored periodically to insure that ambient magnetic field strengths were below 0.5 μT. Embryos were incubated (37.8 ± 0.1°C) for 96 h (developmental stages ranged from 22 to 25 [Hamburger and Hamilton, 1951]) in a water-jacketed incubators (VWR) maintained at $>55\%$ humidity. Embryos of these stages were readily visible with conspicuous blood vessels surrounding them. Incubators were modified to minimize stray magnetic field emissions, and the ambient measurements indicated that magnetic fields were below 0.5 μT at all egg placement positions. Typical geomagnetic and other static fields measured in all of our incubators were ~40–50 μT. During all experiments, embryos were maintained at 37.8°C, except for brief intervals (30–60 s) required to handle the eggs or transfer them to different treatment conditions. During these intervals, embryos experienced room temperature air (~22°C).

Microwave Exposures

Exposures were carried out using a Crawford cell housed within a water-jacketed incubator maintained at 37.8°C. The Crawford cell (model CC110-SPEC, DC to 1,000 MHz, Instruments for Industry, Farmingdale, NY) was mounted vertically on a table using rotary connectors so that it could move freely about the vertical axis. The Crawford cell is a TEM cell with a flat center conductor, which separates its interior into two chambers. Access to each chamber was provided through doors located on opposite sides of the cell. Each chamber was fitted with non-microwave absorbing plexiglass shelves, which were used for sample placement.

Four fertilized eggs were placed in each chamber using holders to position the eggs vertically (narrow side of the egg down, such that the air sac and the embryos were up). The microwave signal was routed from top to bottom in the Crawford cell. All exposures were carried out at a nominal frequency of 915 MHz using a sweep oscillator (HP model 8657B with HP 83522A RF plug-in and a 10-W solid state microwave amplifier (Amplifier Research model 10W1000) as a signal source. Coupling of the

signal to the Crawford cell was accomplished using a double stub tuner (Weinschel Engineering model DS109) to obtain optimum impedance matching. A bi-directional coupler (Narda model 3020) and three power meters (HP432A with HP 478A thermistor mounts) with suitable attenuators were used to monitor the incident, reflected, and transmitted power. For some exposures, superimposed random ELF-EM noise fields (band width from 30 to 90 Hz; 20 μT) were made using a random noise generator built at Catholic University, incorporated into a 35-W audio amplifier. The EM noise fields were applied using a Helmholtz coil pair placed within the microwave exposure incubator, which included the Crawford cell within its uniform magnetic field exposure area (see Litovitz et al. [1997] for more details).

Matching of the Crawford cell loaded with dummy egg samples was performed prior to each exposure episode. Matching was accomplished by adjusting the stubs of the tuner so as to minimize the reflected power. Typically, no further adjustment was needed when the dummy samples were replaced with the experimental samples. Exposures were carried out at either a 3.5- or 5-W incident power level (corresponding to approximate SAR values of 1.75 or 2.5 W/Kg, respectively). Temperature increase within the samples due to RF heating was ~1.5°C at the higher power level (5 W). Chick embryos in intact eggs were exposed eight at a time (four in each side of the Crawford cell) for 30 min, and were then transferred to a nonfield incubator (37.8°C). Control embryos were placed in the same holders outside of the Crawford cell (in the same incubator). One set of embryos was used to assay for Hsp70 induction, while a second set of embryos was used for survival studies.

Hypoxia Survival Studies

Embryos were held at 37.8°C for 1 h following microwave exposure, during which, eggs were windowed to visualize the embryo. A portion of the shell was removed, and the inner shell membrane was drawn back to reveal the embryo. Any unusual eggs (abnormal, bleeding, or incorrect developmental stage) were discarded. This was done to insure that there were no confounding variables as to the cause of death of the embryo. At the completion of the hour, embryos were coded and placed into plastic, air-tight bags (10–12 embryos per bag). Coding was performed by an individual other than the experimenter, which ensured blind evaluation of the results. Air was evacuated using gentle suction, and bags were filled with argon and sealed. An oxygen sensor (model STX70, single gas monitor—Industrial Scientific, Oakdale, PA) placed within the bag confirmed that O_2 levels remained below 1% during the experiment. Temperature was maintained at 37.8°C.

Embryo survival was evaluated every 30 min by observing heart beat. Observations (beating or stopped) were entered into a computer, which provided overall survival percentages for each exposure condition. When approximately 37% of the control embryo hearts were still beating (2.5–3.0 h elapsed hypoxia time), bags were opened to allow re-oxygenation. Only experiments in which between 15 and 45% of the control embryos survived following re-oxygenation were used in the final analysis.

Assay for Hsp70 Induction

At the end of the 30 min, 5-W microwave exposure, eggs were transferred to a control incubator held at 37.8°C. Embryos were removed from the incubator and assayed at 60, 90, 120, 150, 180, 210, and 240 min from the start of the microwave exposure. As a positive control for Hsp70 induction, other embryos were heated by placing the eggs in a water-tight bag and submerging them in a 43°C water bath for 1 h followed by a 2-h wait. Embryos ($n = 4$) were removed from the egg by cutting the major blood vessels near the head and tail region and plucking the embryo out with tweezers. They were then immediately placed into 1 ml of ice-cold chick Ringers solution (0.12 M NaCl, 0.005 M KCl, 0.01M $CaCl_2$), and disrupted by sonication (Model 60 Sonic Dismembranator; Fisher Scientific) for 30 s, followed by centrifugation at 12,000 rpm (4°C) for 10 min. Supernatants were collected and Hsp70 levels were measured by Western blotting. Briefly, 10 μg total protein for each sample were run on a 7.5% SDS–polyacrylamide electrophoresis gel ($n = 7$ gels). Proteins were transferred to a nitrocellulose membrane (Amersham, Piscataway, NJ), which was blocked overnight with 5% non-fat milk. A primary antibody (rabbit anti-chicken Hsp70 [Kawazoe et al., 1999] diluted 1:10,000), and secondary antibody (horseradish peroxidase-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 were quantified using Gel Pro image software (Media Cybernetics, Silver Spring MD).

Statistics

The Fisher's exact test (Instat version 3.0 for Windows 98; GraphPad Software, San Diego, CA) was used for statistical analysis.

RESULTS

The 5 W (SAR ~2.5 W/Kg) microwave exposure induced increases in Hsp70 levels that were quantified by Western blotting. Figure 1 shows a representative Western blot of chick embryo lysates taken at various time points following the start of microwave exposure. Consistently measurable increases in Hsp70 levels (on average, 30% above control) were noted at 3.5–4 h after the start of exposure in most studies, however in some replicates, there were increases in Hsp70 noted as early as 2 h from the start of exposure. Final temperatures measured with thermocouples situated next to the embryos did not exceed 38.8°C. Control (non-exposed) embryos also showed inducible levels of Hsp70. Because they are an embryonic system involved in rapid development, the finding of considerable levels of Hsp70 in otherwise non-stressed controls is not unusual, since they are actively translating proteins needed for growth. In order to confirm the absence of Hsp activation at the temperatures reached during the microwave exposure, other chick embryo samples were heated at 39, 41, or 43 °C for 30 min with a 2.5-h wait before sampling. Lysates were then electrophoresed and blotted to probe for Hsp70 levels (data not shown). At 37 and 39°C, no increase in Hsp70 levels were noted, however, relatively slight elevation of Hsp70 was observed at 41°C (85% over control), and intense banding was seen after 43°C heating. Therefore, even the temperature rise measured during the 5.0-W exposure would not be expected to activate the thermal, Hsp pathway.

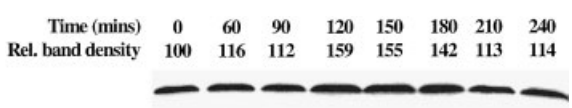

Fig. 1. Shown is a representative blot of Hsp70 levels in chick embryos exposed to microwave radiation. Chick embryos were microwave-exposed for 30 min. Protein samples were taken at the intervals indicated (times shown are from the start of microwave exposure). Control embryos are designated as time 0. Specific Hsp70-positive bands were detected by Western blotting (visualized by chemiluminescence). Resulting X-ray films were analyzed to determine the density of the Hsp70 protein bands, expressed relative to the density of the control band (normalized to 100).

Table I shows results for all microwave− and microwave + noise-induced hypoxia protection studies. Chick embryos were exposed to RF-EM fields with or without superposition of an ELF-EM noise field for 30 min with a 1-h delay prior to initiation of hypoxia. Data for embryo survival, 30 min after re-oxygenation are shown. Chicks that were exposed to RF-EM fields with an incident power of either 3.5 or 5.0 W (SAR ~1.75 or 2.5 W/Kg, respectively) had significantly higher survival rates ($P < 0.01$ and < 0.05, respectively) than controls which were handled identically (except for microwave exposure). When a 20-μT ELF-EM noise field was superimposed, induced protection was eliminated entirely, such that survival of the RF + ELF noise-exposed embryos was indistinguishable from that of non-exposed controls ($P = 1.0$). For all exposure conditions, at least seven replicate experiments with a minimum of 50 total embryos each for control and exposed are reported.

DISCUSSION

The findings presented in the current report indicate that microwave frequency EM fields stress embryonic cells and, without causing a significant temperature increase, enhance Hsp70 protein levels. The small temperature increases observed for the 3.5 (~1.0°C) and 5.0 W incident power RF (~1.5°C), both of which conferred protection against hypoxia, are insufficient to turn on the thermally-induced Hsp pathway [Schlesinger, 1985]. In our experiments, there was an increase, between 20 and 60%, in Hsp70 levels in microwave-exposed embryos (relative to controls). Similar increases in Hsp70 levels following ELF-EM field exposures have been noted by others [Han et al., 1998]. Although considerably smaller than that observed after heat shock [Landry et al., 1982], an average 30% increase appears to be sufficient to induce protection against a secondary stress.

TABLE I. Survival of Chick Embryos Exposed to Microwave Radiation Prior to Hypoxic Stress

Exposure condition	Number of replicates	Control % survival ± SEM[a] (no. of embryos)	RF % survival ± SEM[a] (no. of embryos)	*P* value*
3.5 W	12	29.7 ± 3.3 (74)	51.9 ± 6.0 (79)	< 0.01
5.0 W	14	25.3 ± 3.9 (111)	41.3 ± 4.2 (102)	< 0.05
3.5 W ± 20 μT noise	11	37.8 ± 3.9 (86)	37.4 ± 7.6 (71)	1.00

Chick embryos were exposed to 915-MHz microwave radiation (either 3.5 (SAR ~1.75 W/Kg) or 5 W (SAR ~2.5 W/Kg) incident power) in the absence or presence of 20-μT ELF-EM noise for 30 min (see Litovitz et al. [1997] for additional microwave and noise details), before being placed into hypoxia. Final survival data after hypoxia (shown here) were collected 30 min after re-oxygenation.
*Comparison of RF and control calculated using Fisher's exact test.
[a]SE of the mean.

The finding that even small increases in Hsp70 levels can be biologically important is consistent with the results of Tatar et al. [1997], who showed that increases of only 10–12% in Hsp70 in *Drosophila* were biologically quite significant. We suggest that when induced by athermal EM field exposures, the level of Hsp70 need not be as high as that induced by heat stress, because unlike heating, EM field exposures are capable of stressing the cell without causing damage. Since most of the EM field-induced Hsps are not needed to repair damage from the first stressor, there are more Hsps available to combat damage from exposure to the secondary stress and thus, fewer total molecules are required to effectively pre-condition.

There has been considerable controversy over the existence of athermal EM field induced biological effects. The first finding that EM field exposures could alter stress protein RNA transcript levels was published in 1994 [Goodman et al., 1994]. The same group later reported that Hsp70 protein levels were also altered in EM field-exposed cells [Han et al., 1998]. There have since been several attempts to replicate these results. Pipkin et al. [1999] confirmed Han's findings by demonstrating synthesis of Hsp70 and Hsp27 in cultured HL-60 cells, following a 1-mT exposure to a 60-Hz magnetic field, however, Balcer-Kubiczek et al. [1996] and Morehouse and Owen [2000] were unable to induce stress protein production in the same cell line. Similarly, the induction of stress proteins in *E. coli* by ELF-EM fields was demonstrated by Chow and Tung [2000] but not by Nakasono and Saiki [2000]. Fewer studies have been reported using RF frequency exposures. In *C. elegans*, Daniells et al. [1998], Junkersdorf et al. [2000], and de Pomerai et al. [2000] have reported that by itself, or in combination with heat stress, exposure to microwave radiation can enhance stress protein expression. However, in HeLa and CHO cells, Cleary et al. [1997] were unable to see any microwave-induced increases in stress protein levels. Our findings, reported here, support the conclusion that athermal RF fields can induce biological effects. We have previously addressed possible genetic causes for the replication difficulties noted above [Farrell et al., 1997; Di Carlo and Litovitz, 1999c]. The idea that genetics play a role in EM field responsiveness is consistent with the report of Lin et al. [1999], who noted that even subtle modifications in the promoter region of a gene can affect sensitivity to EM field stimulation.

In the second part of our study, we tested the hypothesis that enhanced Hsp70 would lead to increased cytoprotection following potentially lethal stress. To do this, chick embryos were pre-exposed to RF-EM fields prior to hypoxic stress. As can be seen in Table I, these embryos had significantly higher survival rates after the hypoxic insult than controls. This finding is consistent with previous work in our laboratory, in which 60-Hz magnetic field exposures induced similar protection [Di Carlo et al., 1999a]. In addition, the timing of the induction of Hsp70 observed after RF-EM field exposures is compatible with the timing of hypoxia protection. Hypoxia was initiated 1.5 h after the start of RF-EM field exposure, with reperfusion at 3.5–4 h from the start of exposure. Observed increases in Hsp70, which exceed a 10% increase starting at 2 h from the start of exposure, are well within the time period where protection would be needed. Because the majority of the damage resulting from hypoxia is thought to occur towards the end of hypoxia and

at reperfusion [Van den Hoek et al., 1996], we believe that elevated Hsp70 levels are responsible, at least in part, for the observed hypoxia protection. Others have also shown exposure to EM fields to be protective against subsequent stress. For example, Carmody et al. [2000] reported that 60-Hz magnetic fields could protect rat embryonic heart cells against hypoxic stress, and Grant et al. [1994] and Albertini et al. [1999] have demonstrated that pulsed EM field exposures can protect against ischemia-induced damage in rabbit brain and rat hearts. These protection studies offer further proof that Hsps are induced by athermal EM fields.

We have previously reported that ELF-EM noise fields could inhibit a wide range of EM field-induced biological effects [Mullins et al., 1993; Litovitz et al., 1994, 1997; Farrell et al., 1998; Di Carlo et al., 1999a]. This finding of EM noise inhibition of an EM field-induced biological effect has also been replicated in a number of other laboratories [Lin and Goodman, 1995; Martin and Moses, 1995; Opler et al., 1997; Raskmark and Kwee, 1998]. The fact that concurrent application of EM noise blocks an EM field-induced effect allows EM noise inhibition to serve as a means of verifying that a particular response is caused by the coherent EM field, and not some other variable.

In the current report, we extend this finding to show that the superposition of a 20-μT ELF-EM noise field inhibits hypoxia protection induced by an RF-EM field exposure (Table I). This study was undertaken in order to rule out the possibility of a "hot spot" effect, in which a localized, higher than anticipated temperature rise might have developed during RF-EM field exposure. Although incoherent EM noise fields are thought to block signaling effects, there is no known physical mechanism by which an incoherent EM noise field could cool a hot spot. If the observed hypoxia protection were caused by a hot spot effect, then the addition of an EM noise field would not be expected to alter hypoxia protection. Therefore, the present finding of inhibition of RF-EM field-induced protection by an EM noise field supports the conclusion that the increased protection seen following these exposures is not due to thermal effects.

One can test the hypothesis that EM fields can athermally induce expression of the *Hsp70* gene by either (1) direct *replication* of the reported effect (e.g., enhanced Hsp70 levels) or (2) by testing an *implication* of the hypothesis (e.g., enhanced cyto-protection). We have reported here both replication and implication studies. The results of these studies (both separately and in combination) offer strong support for the hypothesis that athermal EM field exposures induce *Hsp70* expression.

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