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The Effect of 100 mT SMF on Activation of the hsp70 Promoter in a Heat Shock/Luciferase Reporter System

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ABSTRACT

Human exposure to magnetic fields, increased through use of new technologies like magnetic resonance imaging (MRI), has prompted investigations into possible effects of static magnetic fields (SMFs) on cellular processes. However, controversy still remains between many studies, which likely results from a lack of uniformity across experimental parameters, including the length of magnetic field exposure, the strength of the magnetic field, and the cell type or organism under investigation. The purpose of this research was to monitor effects of SMF exposure using real-time luminescence photometry. The study investigated the potential interaction of a 100 mT SMF on a heat shock protein (hsp70)/luciferase reporter construct in stably transfected NIH3T3 cells. Changes in heat shock promoter activation following 100 mT SMF exposure were analyzed and detected as bioluminescence in real-time. Two heat parameters were considered in combination with sham- and 100 mT-exposed experiments: no heat or 1,800 s heat. As expected, there was a significant increase in bioluminescence in response to 1,800 s of heat alone. However, no significant difference in average *hsp70* promoter activation between sham and 100 mT experiments was observed for no heat or 1,800 s heat experiments. Therefore, a 100 mT SMF was shown to have no effect on the activation of the heat shock protein promoter during SMF exposure or when SMF exposure was combined with a heat insult. J. Cell. Biochem. 108: 956–962, 2009.

KEY WORDS: REAL-TIME; HEAT SHOCK; BIOLUMINESCENCE; STRESS RESPONSE

The induction of heat shock proteins (HSPs) in cellular systems following hyperthermal conditions is a well-understood phenomenon [Kiang et al., 1998; Lepock, 2005]. It was first observed by Ritossa and Vonborstel [1964] in Drosophila salivary gland cells. When salivary gland cells were exposed to heat conditions of 42°C or more, a transient and consistent increase in gene expression and protein synthesis was observed. These proteins were thus aptly named "heat shock proteins." Since then, there have been numerous discoveries of new proteins belonging to the heat shock family. Members of the heat shock family are referred to by their molecular weight (i.e., hsp70) with molecular masses ranging from 20 to 110 kDa.

Genes encoding HSPs are found in all cells, both prokaryotic and eukaryotic. They are highly conserved between all cell types, with a 68–70% amino acid identity among eukaryotic cells [Bachelet et al., 1998; Kiang et al., 1998]. Their highly conserved sequence suggests that these proteins play a very important role in cell function and survival. HSPs with molecular weights of 60, 70, 90, and 110 kDa are better understood than others, and are the most highly expressed. In fact, it has been shown that the transcription and translation of these major HSPs is constantly under activation throughout the life cycle under normal physiological conditions, such as cell differentiation or proliferation [Kiang et al., 1998; Santoro, 2000].

The 70 kDa HSP is one of the best understood and conserved proteins in the HSP family. Levels of hsp70 mRNA and protein have been shown to be affected following a variety of external stress stimuli, including food-deprivation [Cara et al., 2005], insulin exposure [Li et al., 2006], high hydrostatic pressure [Kaarniranta et al., 1998, 2000], and electromagnetic field exposure [Goodman and Blank, 1998; Han et al., 1998; Pipkin et al., 1999; Junkersdorf et al., 2000; Tokalov and Gutzeit, 2004; Alfieri et al., 2006; Gottwald et al., 2007] or pulsed magnetic field stimulation [Tsurita et al., 1999]. Under conditions of cellular stress, the heat shock factor (HSF) binds to the heat shock element (HSE) leading to up-regulation of hsp70. The hsp70 is then free to assist in protein folding and

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stabilization, protein characteristics generally compromised during cellular stress [Kiang et al., 1998].

Recently, there has been an interest in the area of magnetic field induced HSPs [Goodman and Blank, 1998; Han et al., 1998; Pipkin et al., 1999; Junkersdorf et al., 2000; Coulton et al., 2004; Tokalov and Gutzeit, 2004; Alfieri et al., 2006; Gottwald et al., 2007; Robertson et al., 2007]. Investigation into a safe and regulated method of elevating hsp70 levels is largely inspired by the clinical benefits of controlled elevated levels of HSPs. Many studies describe the clinical relevance of preconditioning organs and tissues at risk with increased HSP levels [Han et al., 1998; Li et al., 2006; Gottwald et al., 2007; Robertson et al., 2007; George et al., 2008]. Due to the cytoprotective effects of these proteins, it has been shown that increased levels of HSPs are useful in protecting organs and tissues from ischemic injury, metabolic stress, or heat [Han et al., 1998; Li et al., 2006]. The induction of HSPs by magnetic field exposure is considered a safe and controlled way of preconditioning organs to environmental stress often associated with surgery or treatment [Han et al., 1998], with comparatively little or no damage to the tissue. However, there has been contrasting evidence on the consistency of HSP induction by magnetic field exposure. An increase in hsp70 protein levels in response to extremely low frequency electromagnetic fields (ELF-MF) has been shown in mussel immunocytes [Malagoli et al., 2004] and in rat blood [George et al., 2008]. Conversely, it has been shown that there is no change in hsp70 protein levels following ELF-MF exposure in porcine aortic endothelial cells [Gottwald et al., 2007], HL-60, and Girardi heart cells [Bernardini et al., 2007], but an increase in mRNA levels was observed. As with the findings for protein levels, a number of groups have also shown that there is no effect of ELF-MF on *hsp70* mRNA levels [Coulton et al., 2004; Malagoli et al., 2006].

There are fewer studies examining the effect of static magnetic fields (SMFs) on hsp70. Bodega et al. [2005] found that there was no effect on protein levels following exposure to a 1 mT SMF; Abdelmelek et al. [2006] found no change in mRNA after a 128 mT exposure; and Potenza et al. [2004] found that exposure to 200–250 mT induced DNA point mutations in *Escherichia coli*. It is interesting to note that effects begin to emerge as the flux density is increased. There are currently very few studies examining the effect of SMF at or above 100 mT on hsp70 mRNA or protein. In contrast to the lack of consensus regarding the effect of magnetic field on both hsp70 protein and mRNA, several studies have consistently found that ELF-MF, when combined with additional environmental stress such as hyperthermia through exposure to 42°C, produced an observable change in hsp70 levels [Tsurita et al., 1999; Junkersdorf et al., 2000; Tokalov and Gutzeit, 2004]. It is currently unknown whether SMF exposure in combination with hyperthermia affects hsp70 levels. We hypothesized that the combination of hyperthermia and exposure to a 100 mT SMF will affect the rate of the activation of the *hsp70* promoter, measurable through the degree of bioluminescence detected, using a sensitive, real-time, luminescence photometery system. Our approach was to use NIH3T3 cells stably transfected with the *hsp70/luc* construct. Using bioluminescence measurements, we were able to evaluate the effect of a 100 mT SMF alone and in combination with a 42°C thermal stress, both before and during SMF exposure.

MATERIALS

The NIH3T3 stable cell line was a kind gift from Dr. Christopher Contag, Stanford University, California. Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), and Vybrant MTT assay were purchased from Invitrogen (Burlington, ON). The luciferin substrate (D-luciferin) was purchased from Promega (Madison, WI). All other materials were purchased from Sigma–Aldrich Canada (Oakville, ON).

METHODS

CELL CULTURE

The stably transfected NIH3T3 cells were cultured in DMEM supplemented with 10% FBS and antibiotics in a humidified incubator at 37°C, 5% CO_2, and 95% air. Cells were passaged every 2 days in order to maintain a 60–70% confluency. For each experiment, the media was aspirated, and the cell monolayer was washed twice with PBS. A 0.025% solution of Trypsin/EDTA in PBS (2 ml) was added to the monolayer for 1 min. This solution was then removed, and 3 ml of DMEM was added to the culture plate. Cells were mixed with the media using a 1 ml pipette. A volume of 2.4 ml cell suspension was removed from the plate and added to a disposable, plastic cuvette (1 cm × 1 cm). To initiate bioluminescence, 0.75 mg D-luciferin was added to the cuvette immediately before placement in the exposure system. Additionally, cell density was determined before each experiment. A 25 μl aliquot was removed from the remaining 0.6 ml cell suspension in the plate and mixed with an equal volume of Trypan blue (0.4%) in a 1.5 ml Eppendorf tube. A volume of the mixture was transferred to a hemocytometer and viable cells were counted using the Trypan blue exclusion criteria. Typically, 1.5×10^6 to 2.5×10^6 cells were used per experiment.

APPARATUS FOR MEASUREMENT OF BIOLUMINESCENCE

Bioluminescence in luciferin-loaded cell samples was measured using a modified luminescence photometry system, comprised of a photomultiplier tube detector, control electronics, and a desktop computer (Fig. 1—PMT, CE, and PC, respectively) running *Felix32* data collection and spectrometer control software (PTI, London, Ontario). The original commercial sample holder was replaced with a black acrylic enclosure. The enclosure contained an acrylic water bath and a cuvette holder (QC in Fig. 1). As described in detail elsewhere [Belton et al., 2008; Rozanski et al., 2009] a toroidal electromagnet (15 mm pole gap, 16 AWG copper magnet wire, Arnold Engineering) (M in Fig. 1) was held within the water bath (kept at 37 ± 0.1°C or 42 ± 0.1°C) by temperature controlled circulating water. The cuvette was held between the poles of the electromagnet. Water was pumped into the bath via an inflow port (I in Fig. 1) and flowed out through an overflow tube (O in Fig. 1) in order to ensure the electromagnet was completely submerged, but no water would overflow into the cuvette. Bath temperature was monitored with a thermistor and bridge circuit described elsewhere [Carson and Prato, 1996]. The electromagnet was driven by a power amplifier in current mode (7570 AE Techron, Elkhart, IN) and an

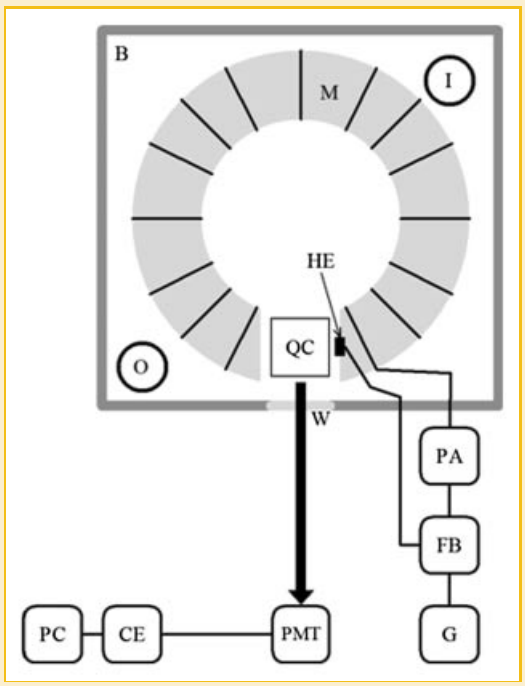

Fig. 1. Exposure apparatus used for hsp70/luciferase experiments. Toroidal electromagnet (M) was placed in a circulating water bath (B) connected to an inflow (I) and an outflow (O) port. Controlled temperatures were maintained at either 37 or 42°C. A disposable cuvette (QC) containing transfected NIH3T3 cells was placed between the poles of the electromagnet. The magnetic field was controlled using a power amplifier (PA), a feedback circuit (FB), and a signal generator (G). The applied SMF was monitored using a Hall Effect sensor (HE). Magnet and cuvette were enclosed in a black box, that blocked out all external light. Photons emitted by cells passed through a small window (W), were collected by the photomultiplier tube (PMT), transformed into an electronic signal via control electronics (CE), and displayed on the PC screen in real-time.

arbitrary function generator (Model 75, Wavetek) (PA and G in Fig. 1, respectively). A custom feedback circuit between the waveform generator and the amplifier was used to monitor and actively control the field (magnetic field error ±0.05 mT) (FB in Fig. 1). Cells were maintained in suspension with a computer-controlled mixer at 3 Hz, with a vertical displacement of 2 mm. The mixer was similar in design to a system described previously [Grundler et al., 1977].

PROTOCOL FOR MEASUREMENT OF BIOLUMINESCENCE

Cells were acclimatized to the exposure apparatus under sham conditions for 300 s prior to any experimental manipulation. Cells in the 0 s thermal stress group remained at 37°C and 0 ± 0.05 mT for the entire 7,200 s experiment. For the group exposed to thermal stress for 1,800 s, the temperature of the water bath was increased to 42°C at a rate of 1°C/min (5 min total) at 300 s and returned to 37°C at 2,100 s. Within each thermal stress group (0 or 1,800 s), there were sham- and SMF-exposed groups. For SMF-exposed groups, the flux density was increased from 0 mT (0–3,900 s) to 100 mT at 3,900 s at a rate of 0.01–0.02 T/s by manual adjustment of the DC signal from the waveform generator and returned to 0 mT at 4,800 s. The sham-exposed group remained at 0 mT for the entire experiment.

The luminescence levels were detected and plotted against time at a sample rate of 0.5 Hz. Experimental groups were as follows: 20 experiments were performed with no heat application (water bath maintained at 37°C), of which 10 included exposure to 100 mT SMF (3,900–4,800 s), and 10 were maintained at 0 mT (sham). A second group of 20 experiments were performed with an 1,800 s heat application of 42°C as described above, of which 10 included exposure to 100 mT SMF (3,900–4,800 s), and 10 were sham exposed.

CELL VIABILITY

Cells were diluted to 3×10^5 cells/ml in phenol red-free DMEM. Two milliliters were exposed to the no heat (37°C for 5,400 s) or 1,800 s heat exposures. These cells were then used in the Vyrbrant MTT assay as per manufacturer's instructions.

TIME SERIES AND STATISTICAL ANALYSIS

Each real-time data series was composed of N individual measures (represented by i) of the sample bioluminescence (B_i) at time points represented by t_i. Sample bioluminescence was in units of photon counts per second. The sample bioluminescence time series was filtered by a moving average operation, where each smoothed bioluminescence value (S_i) was computed using the relation $S_i = \Sigma B_j$ with the summation performed over the range of $j = I–75$ to $j = i + 75$ for values of i between 76 and $N - 75$. Estimates of the instantaneous rate of change (IRC) in the filtered bioluminescence data series ($\Delta S/\Delta t$) in units of photon counts per second squared were computed by numerical differentiation using the relation $\Delta S_{i+1}/\Delta t_{i+1} = (S_{i+1} - S_i)/(t_{i+1} - t_i)$, where i was incremented from 1 to the second last data point $(N - 1)$. Metrics were computed from the data series data by averaging values over a range of time values. Rate of change metrics were computed by averaging values of $\Delta S_{i+1}/\Delta t_{i+1}$ over four time periods: (1) before SMF exposure (3,400–3,700 s), (2) the beginning of the SMF condition, that is, 0 or 100 mT (3,900–4,200 s), (3) the end of the SMF condition (4,300–4,600 s), and (4) after the SMF condition (6,600–6,900 s). The rate of change of the photon acquisition rate was representative of the rate of change of hsp70 promoter activation. All times series calculations were performed with custom software written in C++. The software permitted operation of the time series data in a batch mode, where all time series were processed in sequence by a single computer process. Statistical analysis included two-factor ANOVA (Excel, Microsoft Corp., Redmond, WA). Changes in the rate of bioluminescence detected over time were analyzed and compared between sham- and 100 mT-exposed experiments. Data for the two-factor ANOVA was grouped by SMF condition (sham vs. 100 mT) and time of data analysis (PreField/EarlyField/LateField/PostField) with each ±value representative of the standard error of the mean.

RESULTS

TEMPERATURE MEASUREMENTS

The temperature of the water bath was monitored in real-time throughout each 2 h experiment, using a thermistor and bridge

circuit [Carson and Prato, 1996]. The average water bath temperature was approximately 37°C at basal levels, and was increased to 42°C at t = 1,800 s, in two groups of experiments. The water bath temperature remained elevated until t = 3,600 s. During the 100 mT SMF exposure, the apparent bath temperature increased by approximately 0.1°C, which was attributed to electronic interference of the magnet with the thermistor as described previously [Rozanski et al., 2009]. After the SMF was returned to 0 mT, the bath temperature reading returned to 37°C or 42°C almost immediately depending on the heat condition.

CELL VIABILITY MEASUREMENTS

There was no significant effect of 1,800 s of exposure to 42°C on cell viability as compared to the control cells exposed only to 37°C.

HEAT EXPOSURE DATA AND ANALYSIS

Data collected by the luminescence photometry system was first displayed as a real-time bioluminescence trace, where the number of photons per second (photons/s) detected by the photomultiplier tube were plotted against time, at a rate of one measurement every 2 s. In the raw data trace, the degree of bioluminescence detected over time was dependent on the heat exposure (Fig. 2A). For the group exposed to 37°C only, a non-significant increase in bioluminescence was detected. As expected, a significant increase in bioluminescence was observed following an 1,800 s heat exposure, for both sham and 100 mT groups ($P < 10^{-6}$).

SMF DATA AND ANALYSIS

All experiments conducted in the study were grouped according to two variables: duration of heat exposure and SMF exposure. For each of the heat conditions of 0 or 1,800 s, half of the experiments were sham exposed, and the other half were exposed to 100 mT SMF. Specifically, 20 experiments were performed for each of the heat conditions, of which 10 experiments were exposed to 100 mT SMF, and 10 were sham exposed. A total of 40 experiments were performed in this study. To analyze the effect of a 100 mT SMF in real-time, we looked at the IRC of bioluminescence. Time ranges of IRC measurements have been noted in the methods section and are depicted graphically in Figure 2A.

Groups not exposed to the thermal stress of 42°C had average IRCs of 0.13 ± 0.02 photons/s^2 for Before Field, 0.11 ± 0.02 photons/s^2 for Early Field, 0.12 ± 0.02 photons/s^2 for Late Field, and 0.12 ± 0.02 photons/s^2 for PostField measurements under sham conditions (Fig. 2B). Under 100 mT exposure conditions, the average IRC was 0.12 ± 0.03 photons/s^2 for Before Field, 0.10 ± 0.03 photons/s^2, for Early Field, 0.13 ± 0.03 photons/s^2 for Late Field, and 0.18 ± 0.05 photons/s^2 for PostField measurements (Fig. 2B). There was no statistically significant difference between time ranges for either sham- or SMF-exposed conditions ($P > 0.05$). There was no significant effect of a 100 mT SMF at any of the time ranges ($P > 0.05$) and there was no significant interaction between time of data analysis and SMF-exposure ($P > 0.05$) as determined by a two-way ANOVA. Although there was not a statistically significant difference between SMF- and sham-exposed groups there was a trend towards a higher IRC in the postField time range. Additionally, we plotted the raw bioluminescence values and the IRC against time for the period of time 300 s before and after the moment the SMF was applied (Fig. 3). Qualitative analysis of the graphs (Figs. 3A–D) indicated that there was no effect on either the bioluminescence values or the IRC as the SMF was applied.

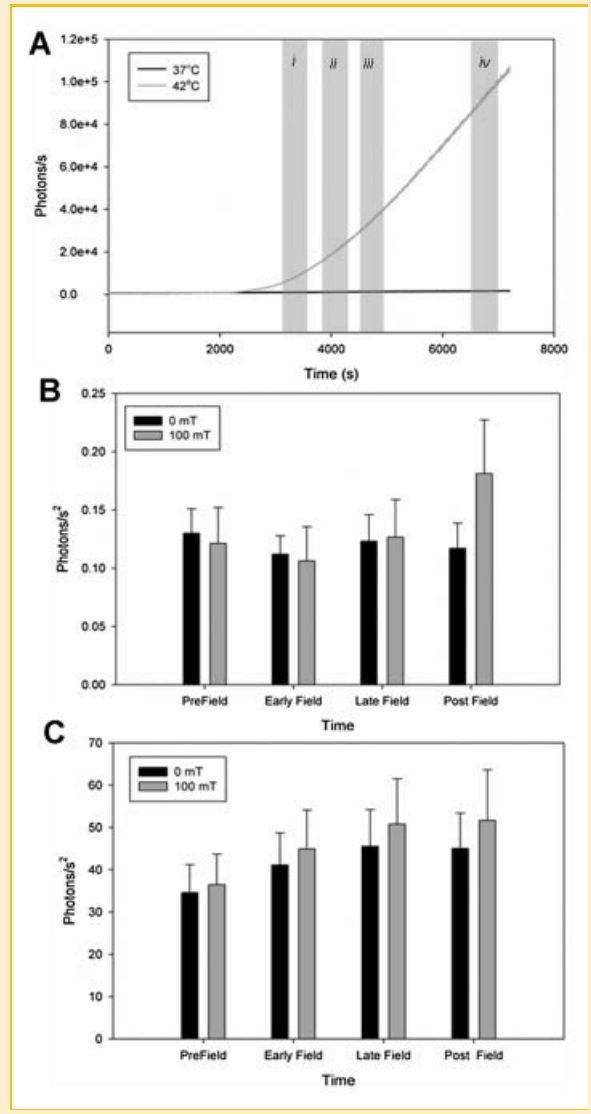

Fig. 2. A: Representative time trace of raw bioluminescence data after exposure to 37°C only or an exposure of 30 min at 42°C. Shaded areas represent the time for each metric (i, PreField; ii, Early Field; iii, Late Field; and iv, PostField). B,C: Effect of a 100 mT SMF on the average instantaneous rate of change for groups exposed to 37°C only (B) or to 30 min at 42°C (C) at different time points. Each bar represents the average of 10 replicates. Error bars represents the s.e.m.

Groups exposed to 42°C for 1,800 s had average instantaneous rates of change of 34.6 ± 6.6 photons/s^2 for Before Field, 41.1 ± 7.6 photons/s^2 for Early Field, 45.5 ± 8.7 photons/s^2 for Late Field, and 45.1 ± 8.3 photons/s^2 for PostField measurements under sham conditions (Fig. 2C). Under the 100 mT SMF condition, the average IRC was 36.4 ± 7.2 photons/s^2 for Before Field, 44.9 ± 9.2 photons/s^2 for Early Field, 50.8 ± 10.7 photons/s^2 for Late Field, and 51.7 ± 12.0 photons/s^2 for PostField measurements (Fig. 2C).

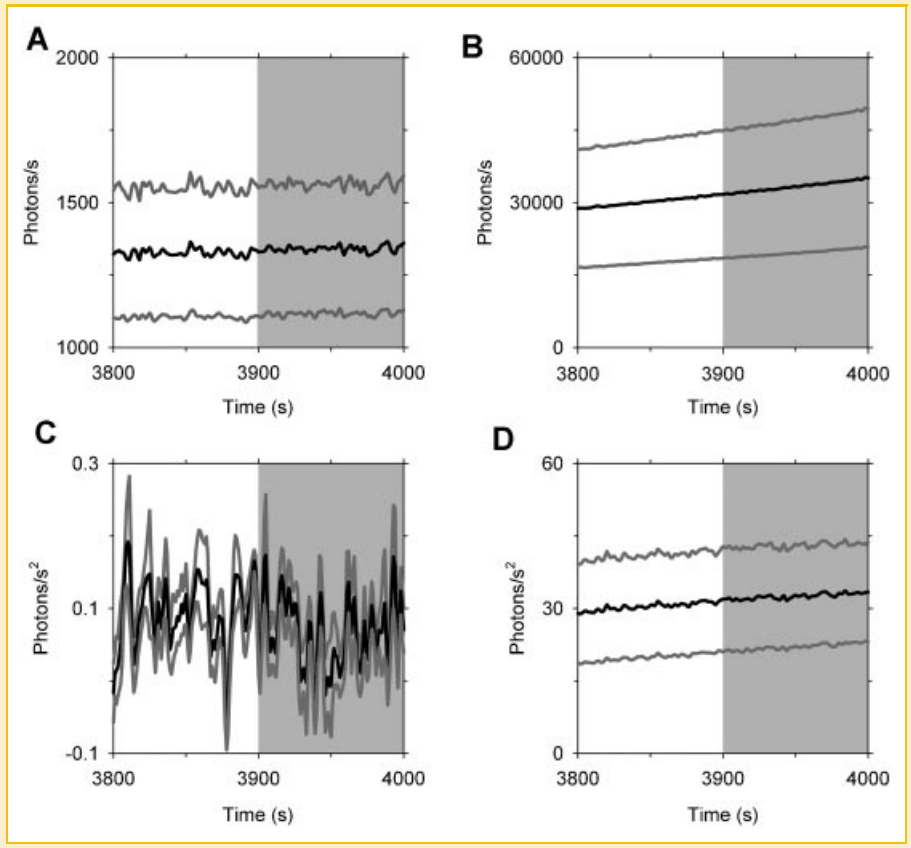

Fig. 3. Effect of the application of a 100 mT SMF on (A) bioluminescence under control conditions (37°C), (B) bioluminescence under hyperthermic conditions (42°C), (C) the IRC under control conditions, and (D) the IRC under hyperthermic conditions. For each graph, the black line represents the average of 10 replicates while gray lines represent the boundaries for s.e.m. Shaded portion of each graph represents area of SMF application.

There was no statistically significant difference between time of data analysis for either sham- or SMF-exposed conditions ($P > 0.05$). There was no significant effect of a 100 mT SMF at any of the time ranges ($P > 0.05$) and there was no significant interaction between time of data analysis and SMF-exposure ($P > 0.05$) as determined by a two-way ANOVA.

DISCUSSION

QUALITY OF BIOLUMINESCENCE DATA AND POTENTIAL TEMPERATURE ARTIFACT

The data collected with the hsp70/luc reporter was reliable and sensitive to changes in heat exposure. The observed increase in bioluminescence following heat was significant and substantial. An increase in hsp70 promoter activation was to be expected following hyperthermia, as shown in other studies [Santoro, 2000; O'Connell-Rodwell et al., 2004; Lepock, 2005]. Due to the nature of the hsp70/luciferase construct, the increase in promoter activation should correspond to a representative increase in bioluminescence production, which is what was observed for all of the heat-exposed experiments. The data from this study was also not corrupted by potential artifacts from SMF-related changes in water bath temperature. No direct influence on water bath temperature was observed before, during or after the application of current to the electromagnet either for the sham or 100 mT experimental runs, as described previously [Rozanski et al., 2009].

EFFECTS OF SMF ON HSP70/LUCIFERASE CONSTRUCT

Under non-hyperthermic conditions we did not observe any significant effect of a 100 mT SMF on the level of bioluminescence produced by the hsp70/luc reporter construct in NIH 3T3 cells. There was however, a non-significant trend towards an increase in the bioluminescence and hence hsp70 production during the postField time measurements (Fig. 2B). These results are similar to those found by Abdelmelek et al. [2006] who also observed a non-significant increase in hsp70 protein using a 1 h/day exposure to a 128 mT SMF, a field strength very close to the one used in this study. This is in contrast to other groups who have shown that SMF significantly affect the levels of hsp70 protein. Potenza et al. [2004] observed that an overnight exposure to SMF ranging in strength from 200 to 250 mT caused point mutations in bacterial DNA. Recently, Tenuzzo et al. [2008] found that a 24 h exposure to a 6 mT SMF decreased the levels of hsp70 protein in human lymphocytes in vitro. It is interesting to note that the main difference between the two studies showing significant results and the studies which found non-significant trends (including this study) is the length of exposure to the SMF. In this study, we exposed the cells to 15 min of SMF and Abdelmelek et al. exposed for 1 h for 5 days. This is in contrast to

Potenza et al. and Tenuzzo et al. who exposed for time frames ranging from overnight to 24 h. This may be an important variable as the cellular changes evoked by SMF may take a significant amount of time to elicit an effect. It has been hypothesized that magnetic fields exert an effect by causing morphological changes in the plasma membrane [Dini and Abbro, 2005]. In fact, it has been shown that a 6 mT SMF results in changes in cell shape and membrane morphology [Chionna et al., 2005]. It is not unreasonable to assume that these changes may require a much longer exposure to the magnetic field to be induced.

When we combined exposure to a 100 mT SMF with hyperthermia (exposure to 42°C for 1,800 s) we did not observe any significant changes in bioluminescence at any of the time points examined. This is in contrast to other groups who have shown that exposure to a magnetic field in conjunction to the stress of hyperthermia synergistically heightens the response of hsp70. For example, Junkersdorf et al. [2000] found that exposure to a 100 μT electromagnetic field in combination with exposure to increased temperatures resulted in an increase in β-galactosidase activity in a lacZ/hsp70 reporter system in *Caenorhabditis elegans*. Tokalov and Gutzeit [2004] found a similar effect in HL-60 cells simultaneously exposed to 30 min of hyperthermia and electromagnetic field while Rodríguez de la Fuente et al. [2009] found an increase in bioluminescence in an hsp70/luc reporter system when an electromagnetic field and hyperthermia was applied for 30 and 20 min, respectively. In contrast to the argument that a long exposure to magnetic fields is required for any observable effect, when magnetic field exposure is combined with the stress of hyperthermia, much shorter exposures seem to be required as can be noted by the above-mentioned examples. This may be because alternative pathways are being recruited. For example, if exposure to magnetic field alone affects membrane morphology, exposure to a combination of magnetic field and heat may activate other pathways involving hsp70 including anti-apoptotic pathways [Schett et al., 1999; Asea, 2003]. Despite the fact that long exposures to magnetic field do not seem to be required in order to elicit an effect when combined with hyperthermia, we did not observe an increase in bioluminescence using an hsp70/luc reporter system. This may be explained by the fact that we were using a SMF while all other groups combining field with hyperthermia have been using an ELF-MF.

CONCLUSIONS AND FUTURE WORK

Based on the results in this study, it was observed that SMF exposure did not significantly affect the rate of hsp70 promoter activation after SMF exposure alone or after the cells had been pre-stressed with an 1,800 s, 42°C heat insult, and allowed to recover for an additional 1,800 s before SMF application. Additional work could be done using different SMF flux densities since a number of studies have found effects at much lower field strengths. Furthermore, in order to validate the hsp70/luciferase reporter system, it would be useful to compare the level of endogenous hsp70 to the level of luciferase produced via the hsp70 promoter reporter system. Such studies could be done under varying heat and SMF exposures in order to ensure the system works under a wide range of experimental conditions.

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