The Proliferative Response of NB69 Human Neuroblastoma Cells to a 50 Hz Magnetic Field is mediated by ERK1/2 Signaling

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Key Words

Magnetic fields • ELF • Neuroblastoma • ERK1/2 • proliferation

Abstract

A number of studies have reported that extremely low frequency magnetic fields (ELF-MF) can modulate proliferative processes *in vitro*; however, the transduction mechanisms implicated in such phenomena remain to be identified. The present study was aimed to determine whether a 50 Hz, 100 μ T MF can induce cell proliferation in the human neuroblastoma line NB69, and whether the signaling pathway MAPK-ERK1/2 (Mitogen-Activated Protein Kinase - Extracellular-Signal-Regulated Kinase 1 and 2) is involved in that proliferative response. The cultures were exposed intermittently or continuously to the MF for a 63-hour duration. The continuous treatment did not induce significant changes in cell proliferation. In contrast, intermittent exposure caused statistically significant increase in the percent of cells in phase S of the cell cycle, followed by a significant increase in cell number. The intermittent treatment also induced an early, transient and repetitive activation of ERK1/2 that could be involved in the

proliferative effects. In fact, both the proliferative response and the repeated activation of ERK1/2 were blocked by PD98059, the specific inhibitor of MEK (ERK kinases 1 and 2). Taken together, the described results indicate that a 50 Hz, 100 μ T MF can stimulate proliferation in NB69 cells by triggering MAPK-ERK1/2 signaling at each of the "On" periods of an intermittent exposure.

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Introduction

There is limited epidemiological evidence suggesting that chronic exposure to weak, extremely low frequency magnetic fields (ELF MF) in residential or occupational environments, could be a risk factor for some cancer types and neurodegenerative diseases [1-4]. On the basis of the epidemiological and experimental evidence, the International Agency for Research on Cancer (IARC) [5] and the National Institute of Environmental Health Science (NIEHS) [6] have classified ELF MF as

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“possible carcinogenic to humans”. Nevertheless, uncertainty persists on this matter and other international bodies, including the International Commission on Non-Ionizing Radiation Protection (ICNIRP), consider that the current evidence on the carcinogenic potential of weak ELF fields is limited and insufficient [7]. The uncertainty is mainly due to the fact that the biological mechanisms underlying the MF interactions at the cellular and subcellular levels are not sufficiently understood.

A large number of studies have investigated the possibility that power frequency MF (50–60 Hz) could influence phenomena involved in cancer promotion [8]. The reported biological responses include changes in cell proliferation [9] cell differentiation [10] or apoptosis [11]. However, the proliferation response has been investigated by a scarce number of studies, most of which applied magnetic flux densities above the values of 500 μ T and 100 μ T proposed by ICNIRP [12] for protection of the workers and the general public, respectively, against exposure to 50-Hz MF. As a whole, the results of these studies have been considered inconsistent and contradictory, since they have reported proliferative effects [13, 14], antiproliferative responses [15–17] or no effect [18]. It has been proposed that such inconsistency could be due to the fact that the MF-induced biological responses are influenced by a number of biological factors, like the genetic characteristics or differentiation status of the cells, or physical factors, like some field parameters or the chronological pattern of exposure [8, 19–24].

The Extracellular-Signal-Regulated Kinase 1 and 2 (ERK1/2) is a member of the family of the Mitogen-Activated Protein Kinases (MAPK) and plays a pivotal role in many physiological and cellular processes [see 25 for a review], including the regulation of cell proliferation [26]. Aberrant ERK1/2 signaling has been repeatedly observed in various cancer types, correlates with worsening of tumour stage and grade and favours metastasis formation [27, 28]. MAPK pathways, including ERK, JNK/SAPK and p38 are activated in response to a number of extracellular stimuli [29]. Among these stimuli, ELF electromagnetic fields have been reported to cause alterations in the MAP-ERK1/2 activation in different cell types [30, 31]. However, a direct relationship between MF-induced activation of ERK and morphological or functional alterations in target cells has not been described so far.

Previous data by our group indicate that intermittent exposure to a 50 Hz, 100 μ T MF could enhance proliferation in NB69 cells from a human neuroblastoma [32–34]. The present study was addressed to confirm and

characterize such a proliferative response, and investigate the signaling events involved in it. The potential participation of the MAPK-ERK1/2 signaling pathway in the MF-induced cytoproliferation was studied by using a synthetic inhibitor of MAPK/ERK Kinase-1/2. The possible involvement of the transcription factor cyclic-AMP responsive element binding protein (CREB) in the proliferative response to MF was also investigated.

The obtained results reinforce our previously reported data and confirm that intermittent exposure to 50 Hz, 100 μ T MF significantly stimulates proliferation in NB69 cells. The magnetic stimulus induces repeated transitory activation of the MAPK- ERK1/2 signaling pathway, occurring early after the onset of each of the exposure cycles of the intermittent treatment. When a specific inhibitor of the MAPK-ERK1/2 pathway was added to the culture medium, the two effects elicited by the MF: the early activation of ERK 1/2 and the proliferative response observed subsequently, were blocked. From this we conclude that activation of ERK1/2 is involved in the molecular mechanisms through which the 50 Hz MF induces a proliferative response in NB69. Additionally, our data show that the field exposure also activates CREB, a downstream target of the MAPK- ERK1/2 pathway. However, such activation is independent of that of ERK, which is indicative that other pathways could be involved in the cellular and molecular response to the MF. As a whole, these results provide a further insight on the mechanisms through which weak, 50 Hz MF could influence cancer-related processes in human cells.

Materials and Methods

Cell culture

The NB69 human neuroblastoma cell line was provided by Dr. M.A. Mena (Hospital Ramón y Cajal, Madrid, Spain). NB69 cells were grown in Dulbecco's Minimum Essential Medium (D-MEM, Biowhittaker, Lonza, Verviers, Belgium) supplemented with 15% heat inactivated foetal bovine serum (GIBCO, Invitrogen, Paisley, Scotland, UK), 4 mM L-Glutamine and 100 U/ml penicillin plus 100 U/ml streptomycin and 0.25 μ g/ml of antimycotic amphotericin B (Gibco BRL, Invitrogen, Prat de Llobregat, Spain). In each experimental run 4.5×10^4 cells ml^{-1} were seeded into 60 mm diameter plastic Petri dishes (Nunc, LabClinics, Barcelona, Spain) and cultured for 3 or 4 days inside an incubator (Forma Scientific, Thermofisher, Waltham, MA, USA) with a 37 °C, 5% CO₂ and 90% relative humidity atmosphere. The culture medium was renewed on day 3 post-plating. When needed, the new medium was supplemented with 20 μ M PD98059 (2'-amino-3'-methoxyflavone; BioSource, LabClinics, Barcelona, Spain), a

specific inhibitor of MAPK/ERK Kinase-1/2 (MEK), which is an upstream kinase of ERK1/2. PD98059 was dissolved in dimethyl sulfoxide (DMSO, Sigma, Madrid, Spain). In a preparatory test, different doses of the inhibitor were assayed. Matched doses of the vehicle, DMSO, were added to controls. The results revealed that the optimal dose of PD98059 was 20 μ M (data not shown).

Magnetic field exposure

NB69 cells were exposed to a 50 Hz, sine wave, linearly polarized magnetic field (MF), at 100 μ T, perpendicular to the growth surface of the cells. The MF exposure set-up has been described elsewhere by Trillo et al., [34]. The MF was created by a wave generator Newtronic Model 200MSTPC (Madrid, Spain) connected to pairs of coils set in a Helmholtz configuration. Currents in the coils were monitored using a multimeter (Hewlett Packard, model 974A, Loveland, CO, USA) and routinely checked by magnetometers (EFA-3, Wandel and Goltermann S.A, Eningen, Germany; EMDEX II, Enertech Consultants, Campbell CA, USA). Two identical pairs of coils were placed inside two magnetically shielded chambers (Amuneal Corp., Philadelphia, PA, USA) made of conetic alloy. The chambers were located inside two identical CO₂ incubators (Forma Scientific, Thermofisher) with a 5% CO₂, 37 °C and 90% humidity atmosphere. The background MF inside the shielded chambers was B_{AC}: 0.04 $\pm$ 0.03 μ T (rms); B_{DC}: 0.05 $\pm$ 0.04 μ T (rms). In each experimental run only one set of coils was energized. The samples in the unenergized set were considered sham-exposed controls. Cell culture dishes were placed in the uniform MF space within the coils for exposure or sham-exposure, with a relatively uniform flux density (100 $\pm$ 0.1 μ T maximum variation) in a region in the middle of the coils where the samples were placed. Both incubators were used alternatively, in a random sequence, for MF exposure and sham-exposure. Cells were exposed to either continuous or intermittent magnetic fields. Two types of intermittence were tested, 5 min On/10 min Off or 3 h On/3 h Off. Both intermittence patterns had been shown to affect different biological systems *in vitro* [19, 23, 32, 34]. All procedures and analysis were conducted blindly for experimental conditions.

Growth response at the end of 63 hours of continuous or intermittent exposure

NB69 cells seeded in Petri dishes were cultured following the general procedure for three days. At the end of this period the culture media were renewed and the cells were exposed to the MF. In each experimental run, five Petri dishes were exposed, whereas 5 other dishes were incubated under identical conditions but remained unexposed (sham-exposed controls). The MF exposure was administered continuously or intermittently over a period of 63 hours. At the end of this exposure and/or incubation lapse, on day 6 post-plating, when the cell confluence in controls was about 80%, cell viability and cell number were assessed through dye exclusion with 0.4% Trypan blue (Sigma).

Analysis of the growth response at the end of a 63-hour lapse of intermittent exposure in the presence or absence of PD98059

The NB69 cells were cultured under standard conditions for three days. At this time, the media were renewed and 60 min prior the MF- or sham-exposure onset, 20 μ M PD98059, the ERK1/2 inhibitor, was added to the new media. Two groups of 10 Petri dishes, 5 with inhibitor and 5 without it, were distributed in each of the two sets of coils placed inside the MF-shielded chambers. The MF-treated group was submitted for 63 hours to a 3h On / 3h Off exposure cycle. Four experimental conditions were tested: a) sham-exposure in the absence of inhibitor (controls), b) treatment with inhibitor only, c) exposure to MF only, and d) MF exposure in the presence of inhibitor. At the end of the 63-hour experimental period, on day 6 post-plating, cell viability and cell growth were determined by Trypan blue dye exclusion.

Proliferation analysis by immunostaining with Bromodeoxyuridine

Cells were seeded on circular, 12 mm diameter coverslips (Hirschmann Laboratories, Eberstadt, Germany) placed into the Petri dishes. Following the general procedure, including medium renewal at day 3 and subsequent MF- or sham-exposure, the dishes were incubated for four or five days. At these times, 5 μ M 5-bromo-2'-deoxyuridine (BrdU, Sigma) a marker for cells in the S-phase of the cycle, was added to the media. After 21 h of intermittent exposure to the MF (3 h On/ 3 h Off) or sham-exposure (day 5 or 6), the amount of BrdU incorporated by the cells was quantified by immunofluorescence using 1:20 monoclonal antibody anti-BrdU (Dako, Barcelona, Spain). The samples were analyzed through fluorescence microscopy (Nikon Eclipse TE300; Melville, USA) and Computer-Assisted Image Analysis (Analy-SIS, GMBH, Munich, Germany). In each of 4 experimental replicates, 3 coverslips were analyzed per experimental group. The percent of BrdU-positive cells and the total nuclei (Hoechst 33342 fluorescence dye, Bisbenzimidazole, Sigma) were recorded in 15 randomly selected fields per coverslip. In each experimental replicate, a total of about 4000 - 4500 cells per experimental group were evaluated.

Immunocytochemical analysis of ERK1/2- and CREB-activation in response to short-term exposure to the field

To examine the time course for activation of ERK1/2 and CREB, in terms of rates of positive cells for phospho-ERK1/2 (pERK1/2) or phospho-CREB (pCREB), samples were incubated for four days, following the general procedure, on 12-mm diameter coverslips placed into the Petri dishes. For ERK1/2 activation, samples were exposed to MF and/or incubated for 15, 30, 60 or 120 minutes. In each experimental replicate, half of the samples were pretreated with 20 μ M of the ERK1/2 inhibitor, PD98059, for one hour prior the MF- or sham-exposure. At the end of treatments, activation of ERK1/2 was characterized by immunocytochemistry and Computer-Assisted Image Analysis. The primary and secondary antibodies: antiphospho-specific ERK1/2 (1:100; BioSource) and goat anti-rabbit IgG anti-

body (1:500) conjugated with the fluorophore Alexa Fluor 488 (Molecular Probes, Invitrogen, Prat de Llobregat, Spain) respectively, were used. For CREB activation, samples were exposed to MF and/or incubated for 30, 60 or 120 minutes. The polyclonal anti-pCREB antibody (1:500, Upstate, Cell Signaling Solutions, Barcelona, Spain) and a biotinylated secondary antibody (1:100) were used. Immunostaining was enhanced through the ABC method (Vectastain ABC kit; Vector Laboratories, Atom S.A, Barcelona, Spain), and revealed with 3'3'-diaminobenzidine (DAB, Sigma).

In each replicate 3 coverslips per experimental group were studied. The percentages of immunoreactive cells were determined in at least 15 randomly selected fields per coverslip. The percents of pERK1/2- or pCREB-positive cells, were calculated over the total number of cells, revealed by Hoechst or methyl green counterstaining of the nuclei, respectively. In each of four replicates, a total of about 4000 - 4500 cells per experimental group were evaluated.

Western blot analysis for phosphorylated ERK1/2 and CREB

The phosphorylation of ERK1/2 and CREB was assayed by Western blot in samples exposed and/or incubated for 30, 60 or 120 minutes at day four post-plating. Briefly, the cells were lysed in buffer containing 10 mM Tris-HCL (pH 7.6), 100 mM potassium chloride (KCL), 1 mM EDTA, 1mM Dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml Leupeptin, 5 µg/ml Pepstatin A, 100 mM NaF, 20 mM β – glycerolphosphate, 20 mM sodium Molybdate, 0.5% Tritón X-100 and 0.1% SDS. The protein concentration was determined using the Bradford's colorimetric method. For blotting, equal amounts of protein (40-50 µg) were run in 10% SDS/PAGE gels and transferred to a nitrocellulose membrane. Membranes were blocked (5% dry milk in Tween-TBS) and incubated with the primary polyclonal antibodies anti pERK1/2 (1:1000; BioSource) or anti pCREB (1:1000, Upstate Cell Signaling Solutions). Then, the membranes were incubated with horseradish peroxidase-conjugated, Anti-Rabbit IgG secondary antibody (1:5000, GE Healthcare, Little Chalfont, Buckinghamshire, UK). Anti β-actin (Sigma) was used as loading control. The blots were developed through enhanced chemiluminescence (ECL Advance Western Blotting Detection Kit, GE Healthcare) and exposed to X-ray film. The exposures of the X-ray film were done to be in the linear response region of the film. Densitometric analysis of the protein bands was performed using a computer imaging device and accompanying software (Quantity-One, Biorad, Munich, Germany). Western blot analyses for pCREB-expression in the presence or absence of 20 µM PD98059 were also conducted.

Short-term ERK1 /2-activation assays at different on / off cycles of intermittent exposure

On day 3 post-plating, cells seeded on 12 mm diameter coverslips placed into Petri dishes, were pretreated for 60 minutes with ERK1/2 inhibitor or with the corresponding vehicle, and MF-exposed or sham-exposed intermittently, as described above. The samples were analyzed at 30 minutes from the onset of different On or Off periods: a) at 18:30 h, corresponding

Fig. 1. Effect of 63 hours of continuous (Cont) or intermittent (3 h On / 3 h Off or 5 min On / 10 min Off) exposure to a 100 µT MF, on the number of cells. Means ± SEM of four experimental replicates per MF exposure pattern; normalized values over the respective sham-exposed controls (5 MF-exposed dishes per replicate; 20 exposed dishes per exposure pattern). *: p<0.05; **: p<0.01 (Student's *t* test).

to the On period between 18-21 hours; b) at 21:30 h, corresponding to the Off period between 21-24 hours, and c) at 60:30 h, corresponding to the last On period, between 60-63 hours. At the end of these 30-minute intervals, the samples were immunocytochemically assayed for phosphorylated ERK1/2, and the percent of pERK1/2-positive cells was quantified by Computer-Assisted Image Analysis. In each experimental replicate 3 coverslips were studied per experimental group, and 15 randomly selected microscope-fields were analyzed per coverslip. In each of three replicates per time point, a total of about 4000 - 4500 cells per experimental group were evaluated.

Statistical analysis

The data were normalized over the respective control samples and the values were presented as means ± SEM of at least three independent experimental replicates. The data corresponding to treated groups and their respective controls were compared by two-sample Student's *t* test. The multifactorial one-way analysis of variance, ANOVA, was used to assess differences between multiple sets of data. The limit of statistical significance was set at p<0.05.

Results

Proliferative response after continuous or intermittent exposure to MF

The results summarized in Fig. 1 show that 3 h On/ 3 h Off and 5 min On/ 10 min Off intermittent exposure to the MF induced similar, statistically significant increases

in the number of cells. As the 3 h On/ 3 h Off intermittence induced the most consistent response, this exposure pattern was selected to be used in subsequent experiments. On its part, continuous exposure did not change the cell number significantly. Also, none of the three MF exposure conditions changed significantly the cell viability rate (data not shown).

As shown in Fig. 2, after 42 h of intermittent (3 h On/ 3 h Off) exposure, a statistically significant increase in the number of BrdU-positive cells was observed (42.9% over sham-exposed controls). By contrast, no significant changes in BrdU incorporation were detected after a 63-h exposure to the same MF parameters. This indicates that MF-induced enhancement of DNA synthesis occurs at least 20 hours prior to the increment in cell number, observed at the end of the 63-hour exposure.

Proliferative response to the MF in the presence of PD98059

The data represented in Fig. 3 show that a 63-hour treatment with the ERK1/2 inhibitor PD98059, significantly decreased the number of cells with respect to untreated controls. An equivalent decrease in the cell number was obtained after 63 hours of intermittent exposure to the MF in the presence of PD98059, showing that the ERK1/2 inhibitor can completely block or revert the MF-elicited proliferative response. None of the applied treatments affected the cell viability rate. These results indicate that ERK signaling could be implicated in the cytoproliferative effect of the MF, and are suggestive of a potential activation of ERK1/2 by the magnetic stimulus.

Short-term activation of ERK1/2 by MF exposure

The short-term effect of MF on the ERK1/2 activation, expressed as the percent of cells displaying ERK1/2 phosphorylation within the first 15 - 120 min of the first 3-h On interval of exposure, was studied by immunocytochemical (Fig. 4) and Western blot analyses (Fig. 5). After 30 or 60 min of field exposure, the percent of pERK1/2-positive cells was significantly increased with respect to that of controls (Fig. 4). These MF-induced responses were inhibited by PD98059. However, the inhibitor alone did not change the rate of pERK1/2-positive cells at 30 or 60 min. The MF exposure time of 15 min seems to be too short to induce changes in the percent of pERK1/2-positive cells, whereas at 120 min a significant decrease of about 19% below controls ($p < 0.001$) was observed. At this

Fig. 2. A) Quantitative analysis of BrdU-positive (BrdU+) cells in samples intermittently exposed (3 h On/3 h Off) to the MF and/or incubated for 42 h or 63 h. Data normalized over sham-exposed controls, and expressed as Means $\pm$ SEM of 4 experimental replicates. ***: $p < 0.001$ (Student's t test). B) Representative example of immunostaining in controls and MF-exposed cells. BrdU+ cells (upper panels) and Hoechst nuclear stain (lower panels). The BrdU+ rate is calculated as the number of cells incorporating BrdU divided by the number of Hoechst-stained nuclei (total cell number).

Fig. 3. PD98059-mediated inhibition of the cytoproliferative response induced by a 63-hour intermittent exposure to MF. Means $\pm$ SEM of four experimental replicates. Normalized data. **: $p < 0.01$; ***: $p < 0.001$ (ANOVA and Student's t test).

Fig. 4. A) Short-term effect of MF exposure on the rate of p-ERK1/2 positive cells, analyzed by immunocytochemistry and computer-assisted image analysis in the first 15-120 min of exposure to MF, the kinase inhibitor PD98059, or their combination. Means $\pm$ SEM of 4 experimental replicates. Data are normalized over the corresponding sham-exposed control groups. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ (ANOVA and Student's t test). B) Representative examples of p-ERK1/2 immunostained cells (upper panels) after 30 min of treatment (MF or MF + PD98059) and/or incubation (control). All nuclei were stained with Hoechst (lower panels).

time, the treatment with PD98059, alone or in combination with MF, induced equivalent, statistically significant decreases in the rate of pERK1/2-positive cells.

Immunoblot analyses (Fig. 5) show that at 30 min, the MF exposure induced a significant increase in pERK1/2 expression, whereas at 120 min a significant decrease in pERK1/2 was observed. These results reinforce those obtained by immunocytochemical analysis at the same time intervals of 30 and 120 min.

Sequential intervals of ERK1/2 activation through the On / Off exposure cycle

To investigate the chronological pattern of activation of the extracellular signal-regulated kinase 1

Fig. 5. Phosphorylated-ERK1/2 expression after 30 and 120 minutes of MF exposure and/or incubation was quantified by Western blot analysis. β -actin was used to ensure equal protein loading. A) Phospho ERK1/2 band intensity values normalized over the corresponding sham-exposed controls; Means $\pm$ SEM of 4 experimental replicates. *: $p < 0.05$; ***: $p < 0.001$ (Student's t test). B) Representative blots at 30 and 120 min of exposure.

Fig. 6. Percent of p-ERK-positive cells at 30 minutes of the onset of two "On" and one "Off" intervals of the intermittent exposure to the MF. Significant activation of ERK1/2 was observed only during the "On" intervals of the exposure cycle. Immunocytochemistry and computer-assisted image analysis. Normalized values. Means $\pm$ SEM of three experimental replicates. ***: $p < 0.001$ (Student's t test).

Fig. 7. A) Short-term effect of the MF on the percent of p-CREB positive cells. Immunocytochemistry and computer-assisted image analysis. Normalized data. Means $\pm$ SEM of three independent replicates (at 30 and 120 min) or four independent replicates (at 60 min). ***: $p < 0.001$ (Student's t test). B) Representative examples of immunocytochemical staining of p-CREB in sham-exposed controls and MF-exposed samples (30 min). The cytoplasm of p-CREB positive cells is brown stained. All cell nuclei were stained with methyl green.

and 2 during the intermittent exposure to the MF, three intervals (two On and one Off) were selected, and the pERK1/2 response was analyzed at 30 min after the intervals' onset. As shown in Fig. 6, equivalent, statistically significant increases in the percent of cells expressing pERK1/2 were obtained at the two studied "On" intervals (18 -21 h and 60 - 63 h) of MF exposure. By contrast, no significant changes with respect to controls were observed in the ERK1/2 activation during the "Off" interval. These results indicate that a sequential, transitory activation of ERK1/2 takes place during the "On" intervals along the intermittent exposure to the field. This, together with the above described results suggests that the cytoproliferative response induced in NB69 by intermittent exposure to MF could be mediated by a cyclic activation of MAPK/ERK1/2 pathway.

Short-term activation of CREB by MF

ERK1/2 is among the different signal pathways involved in the activation of CREB. Thus, the subsequent step of the study focused on the MF-effects on CREB activation. The pCREB expression was quantified by immunocytochemical analysis and Western-blot. As shown in the immunocytochemical staining analysis (Fig. 7A) a significant increase in the percent of cells expressing pCREB was detected at 60 min of MF exposure. However, no significant differences with respect to controls were observed at 30 min of exposure nor at 120 min for the immunocytochemical staining. The Western blot data of pCREB expression (Fig. 8A, B) showed also a significant increase in pCREB at 60 min and no effect at 120 min of exposure. Thus, the immunoblot data of pCREB expression at 60 and 120 min (Fig. 8A, B) reinforce the immunostaining results obtained at

Fig. 8. Expression of phosphorylated-CREB at 30 and 120 min of MF exposure and/or incubation. A) Phospho CREB band intensity values normalized over the corresponding sham-exposed controls; Means $\pm$ SEM of three experimental replicates. *: $p < 0.05$ (Student's t test). B) Representative blots at 60 and 120 min of exposure. β -actin was used as loading control.

Fig. 9. Effect of a 60-min exposure to the MF on the expression of p-CREB, in the presence or absence of the ERK1/2 inhibitor (INH). (A) Phospho CREB band intensity values normalized over sham-exposed controls. Means $\pm$ SEM of 4 experimental replicates. *: $p < 0.05$; **: $p < 0.01$ (Student's t test). (B) Representative immunoblot; β -actin was used as loading control.

the same time intervals, and confirm that MF exposure induces transient activation of CREB.

Short-term effect of MF on the activation of CREB in the presence of the ERK1/2 inhibitor

To analyze whether CREB activation could be mediated by the MF-induced activation of MAPK ERK1/2, NB69 samples were MF-exposed for 60 min in the presence or absence of the specific ERK1/2 inhibitor, PD98059. This time lapse was chosen on the basis of the above described result, that a 60-minute MF exposure induces significant activation of CREB. The data summarized in Fig. 9 confirm that short-term exposure to MF significantly increases pCREB expression. An equivalent, statistically significant response was obtained after MF exposure in the presence of the ERK1/2 inhibitor, indicating that the MF-induced activation of CREB is independent of, or is not involved in, the mechanism underlying the MF effect on the activation of the MAPK-ERK signaling pathway. The present data do not allow us to determine whether or not the MF-induced

cytoproliferative response is mediated by the activation of CREB. Nevertheless, the observed activation of CREB raises the possibility that other pathways besides of MAPK-ERK, could be involved in the cytoproliferative effect of the field.

Discussion

The herein results show that 63 hours of continuous exposure to a 50 Hz, 100 μ T MF did not induce significant changes in the proliferation of the human neuroblastoma cell line NB69. In contrast, intermittent exposure to the same MF parameters significantly increased cytoproliferation in NB69 through activation of the MAPK-ERK1/2 signaling pathway. At 42 hours of intermittent exposure, a significant increase was observed in the percent of cells in S-phase, of DNA synthesis, which is consistent with the increase in the cell number obtained 21 hours later, at the end of the 63-hour exposure period. These data confirm previous results from our group [32-34] and highlight the importance of intermittency in the functional changes induced by weak ELF-MF in cellular biosystems. The intermittent treatment also induced early, transient and cyclic activation of ERK1/2, which peaked at the "On" intervals of field exposure. This transient activation of ERK1/2, as well as the cytoproliferative response observed at the end of the 63 hours of exposure, were blocked by the specific inhibitor of MEK1/2, PD98059. As a whole, these results provide new evidence that *in vitro* exposure to weak MF can induce changes in human cancer cells. The effect, which initially could take place at the membranal level, might be transduced through the ERK1/2 signaling pathway and result in promotion of cytoproliferation.

Only a small number of studies focussing on bioeffects of power-frequency MF have tested magnetic flux density values below the safety levels recommended by ICNIRP for occupational protection against short term damage (500 μ T for $f = 50$ Hz). These studies have reported a heterogeneous variety of effects that, taken together, have been often described as contradictory [13-15, 17, 18]. However, it is generally admitted that the elicited cellular responses are dependent on a number of biological and physical parameters. For instance, the intermittency of the signal has been proposed as a critical factor in genotoxicity-related responses on specific cell types [19, 23, 35]. Such view is strongly supported by the present results, which show that an

intermittent exposure, but not a continuous one, can induce proliferation in the NB69 cell line. The current experimental evidence is insufficient to explain why these cells do not seem to be responsive to the continuous exposure. However, our data could be indicative that: 1) in the middle/long term, the cells might develop adaptive mechanisms against the effects of physical stressors as MF, and 2) the effectiveness of such potential mechanisms could be challenged by discontinuous and repeated MF exposure. This point can be relevant, since in real life conditions, the occupational and residential exposures to MF usually follow cyclic, circadian patterns.

At first glance, the proliferative response observed after 63 h of intermittent MF exposure, could be interpreted as mediated by an unspecific stimulus involving the MAPK-ERK1/2 signaling, since the specific inhibitor of this pathway, PD98059, significantly decreased cytoproliferation, both in the presence and in the absence of the MF stimulus. However, during the intermittent “On” intervals of field exposure, ERK1/2 activation occurred, and peaked at 30 minutes of the MF onset. This activation, which was also blocked by PD98059, was not detectable at the “Off” periods of the exposure cycle. This indicates that, in fact, ERK1/2 is specifically involved in the cytoproliferative response over the 63 hours of On / Off cyclic exposure to the field. ERK1 / 2 signaling has been reported to intervene in cell proliferation and cell survival processes [27, 36], and abnormal activation of this pathway could contribute to the development of different pathologies, such as cancer [37].

The ERK transitory activation induced by the MF during the 3-hour “On” interval was not observed at 15 min of exposure, and was significantly reduced at 120 min of exposure. So, the time interval at which the response is analyzed can be a critical factor to the detection of the MF-induced molecular effects, as proposed by other authors [21].

On the other hand, it has been suggested that weak ELF MF could elicit a stress response, similar to that induced by other stress factors [38]. An essential aspect of the stress response is represented by stress-dependent MAPK cascades, with the ERK1/2 as central elements in cells of both, vertebrate and invertebrate organisms [25]. Goodman et al., 2009 [39] have reported that intermittent electro-magnetic fields of 60 Hz and 8 μ T can accelerate tissue repair in *Planaria* by activating the ERK1/2 pathway. Also, ERK1/2 activation has been reported in HL-60 cells after 10 - 30

min of exposure to a 60 Hz, 100 μ T MF. This MF treatment also induced cytodifferentiation in the same cell line [31]. One central question concerning ERK1/2 signaling is how activation of a single protein kinase can elicit different cellular outcomes. It has been reported that differences in the duration, magnitude and subcellular compartmentalization of ERK activity, can generate variations in signaling output that regulate cell fate decision [40, 41]. It is possible that the chronological pattern of ERK1/2 activation by the cyclic exposure to the MF is also critical to the proliferative response of NB69 cells. In addition, in the 3 h On/3 h Off intermittency, the effective exposure time corresponds to 50% of the 63 hours of MF treatment. Perhaps repeated stimulation up to approximately 30 hours of effective exposure could be necessary for triggering the molecular mechanism that activates ERK1/2 and leads to increased proliferation. We have not investigated whether ERK1/2 is involved in the cytoproliferative response induced by the 5 min On / 10 min Off cycle of MF exposure. However, in the 3/3 hours exposure cycle, ERK1/2 activation was not observed at 15 minutes of the MF onset, and peaked within 30 min after exposure. Thus, it is possible that under the intermittency pattern with shorter exposure intervals (5 min On), pathways other than ERK1/2 could mediate in the MF-induced proliferative response.

There is general consensus that the primary target of weak, ELF MF may be located at the cell membrane. There, signal transduction pathways would be triggered through a complex program of transcriptional events involving activation of transcription factors. On the basis of these premises, the epidermal growth factor receptor (EGFR) has been proposed as one potential target of 50 Hz MF [42-44]. Also, activation of transcription factors associated to ERK activation has been described in human leukaemia cells HL-60, in human breast cancer cells MCF-7, and in rat fibroblast 3Y1, exposed to a 60 Hz, 100 μ T MF [31]. The transcription factor CREB represents a crucial integrator of numerous signals from cytoplasmic kinase cascades, including ERK1/2, and is directly involved in regulating the transcription of genes that control cell proliferation and survival [45]. CREB has also been implicated in oncogenesis, as it has been found overexpressed and constitutively phosphorylated in a number of human cancer types, including acute myeloid leukaemia and non-small cell lung cancer [46]. It has been reported that in HL60 cells, treatment with a 50 Hz, 100 μ T MF can cause CREB-DNA binding activation, mediated by

intracellular Ca^{2+} signaling, but independent of ERK, PKA, PKC or p38-MAPK [47]. In the present study, the intermittent exposure to MF stimulated CREB activation, both in the absence and in the presence of the MEK inhibitor, which confirms that the MF-induced activation of CREB is not mediated by the ERK1/2 pathway. Therefore, a potential involvement of different signal transduction pathways, including Ca^{2+} signaling, in the proliferative response induced by the MF cannot be ruled out. In fact, calcium signaling has been described as a protecting factor against MF-induced damage in neuroblastoma cells [48]. The importance of Ca^{2+} in the cellular response to ELF fields has been investigated and reviewed by a number of authors [49-53].

The present results contribute to deepen the knowledge of the mechanisms involved in the proliferative response induced by exposure to weak, power frequency magnetic fields on already initiated human cells. From the herein reported data it cannot be directly inferred that those MF are potentially harmful to humans, although the possibility that malignancy resulted from an accumulative effect of chronically

repeated exposure can not be ignored. In any case, these results contribute to the characterization and understanding of factors underlying the biological effects of these MF. A more complete understanding of these mechanisms would help to define the role of ELF-MF in the complex process of tumour progression. Such knowledge would provide appropriate basis for regulations that warranted efficacious protection of the general public and the workers against potential harmful effects of power frequency MF.

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