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An Incoherent Magnetic Field Inhibited EGF Receptor Clustering and Phosphorylation Induced by a 50-Hz Magnetic Field in Cultured FL Cells

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Key Words

50 Hz magnetic field • Noise/incoherent magnetic field • Receptor • Clustering • Phosphorylation

Abstract

Previously, we found that exposure to a 50-Hz magnetic field (MF) at 0.4 mT could induce epidermal growth factor (EGF) receptor clustering in Chinese hamster lung (CHL) fibroblast cells and superposition of an incoherent MF with the same intensity could inhibit the effect. In the present experiment, we investigated the effects of 50-Hz MF exposure at different intensities on EGF receptor clustering and phosphorylation in human amniotic cells (FL), and explored the interaction effect of an incoherent MF. Clustering and phosphorylation of EGF receptors on cellular membrane surface were analyzed using immunofluorescence assessed by confocal microscopy and western blot technology, respectively. EGF treatment served as a positive control. The results showed that, compared with sham exposure, exposure to a 50-Hz MF at 0.1, 0.2 or 0.4 mT for 15 min could significantly induce EGF receptor clustering and enhance phosphorylation on tyrosine-1173 residue in FL cells, whereas exposure to a 0.05 mT field for 15 min did not caused a significant effect. Exposure to an incoherent MF (frequency range between 30 to 90 Hz) at 0.2 mT for the same time neither induced EGF receptor clustering nor enhanced phosphorylation of EGF receptor in FL cells. When superposed, the incoherent MF at 0.2 mT completely inhibited EGF receptor clustering and phosphorylation induced by a 50-Hz MF at 0.1 and 0.2 mT. However, the incoherent MF could not completely eliminate the effects induced by a 0.4 mT 50-Hz MF. Based on the results of this experiment, we conclude that membrane receptors could be one of the main targets where extremely-low frequency (ELF) MF interacts with cells, and the intensity threshold, in the case of EGF receptors, is between 0.05 and 0.1 mT. An incoherent MF could completely inhibit the effects induced by an ELF-MF of equal or lower intensity.

Introduction

Use of electricity has progressively increased the environmental levels of extremely-low frequency electric

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and magnetic fields (ELF-EMF). Evidence is accumulating that exposure to ELF-EMF could produce deleterious biological/health effects. Especially, some early reports indicated that ELF-EMF, such as those from electric power transmission and distribution lines, is associated with increased risks of childhood leukemia, cancer of the nervous system, lymphomas, and breast cancer [1-6]. Following two pooled analysis of childhood leukemia studies [7, 8] that indicated a doubling of risk at exposure above 0.3/0.4 μT, the International Agency for Research on Cancer (IARC) has classified ELF-EMF as a possible human carcinogen 2B [9]. On the other hand, potentially health beneficial effects of ELF-EMF have also been reported and applied in medical treatments [10-12]. However, the mechanism of ELF-EMF biological effects is still unknown. Exploring the initial interaction loci between ELF-EMF and biological systems, such as the signal transduction processes in cells, would be an important step in discovering the molecular mechanisms of bioeffects. Nie et al. [13] found that ELF-EMF induced mitogen-activated protein (MAP) kinase (Erk1/2) activity. Exposure of B-lineage lymphoid cells to a 60-Hz EMF could stimulate Lyn kinase and lead to activation of protein kinase C (PKC) [14]. Our previous studies showed that exposing Chinese hamster lung (CHL) fibroblast cells to a 50-Hz magnetic field (MF) at 0.4 mT could phosphorylate and activate the stress-activated protein kinase (SAPK) and P38 mitogen-activated protein kinase (P38 MAPK) [15, 16]. Although these is evidence that ELF-EMF exposure may activate signal transduction pathways, as yet, the loci where EMF transfers into biological signals and transduction of biological signals into cellular molecular processes are unknown. Luben [17] pointed out that since low-energy EMF (pulse and direct current) has little energy to directly traverse the cell membrane, it is possible that it may modify the existing signal transduction processes in cell membrane, thus producing both transduction and biochemical amplification of the effects of the field itself. There is some evidence indicating that EMF affects intra-membrane protein distribution [18], phosphorylation [19], structural and biophysical changes in membranes [20], and desensitizes 5-HT(1B) receptor in brain [21]. Based on these data, we hypothesized that membrane proteins, especially receptors are the initial target sites of ELF-EMF. Previously, we found that exposure to a 50-Hz MF at 0.4 mT could induce EGF receptor clustering in Chinese hamster lung (CHL) fibroblast [22]. In the present experiment, we investigated the possible effects of a 50-Hz MF on receptors clustering and phosphorylation, and explored the intensity threshold of the MF on human amniotic cells.

It is now clear that exogenous ELF-MF can cause biological effects. But, how organisms or cells respond to exogenous EMF, which is 100–1000 times weaker than the random thermal noise fields generated by thermal motion of ions in and around the cells is still a puzzle. In order to explain the phenomenon, Litovitz et al. [23, 24] have proposed the temporally and spatially coherent hypothesis of EMF. It states that living cells distinguish thermal noise fields from external EMF because thermal noise fields are temporally and spatially incoherent whereas external EMF is coherent. When exogenous fields are both spatially and temporally coherent, they would induce biological effects. Effects of a spatially and temporally coherent MF would be interfered by superposing a spatially coherent but temporally random incoherent ('noise') MF. In the present experiment, we also investigated whether incoherent MF could interfere receptor clustering and phosporylation induced by a 50-Hz MF. We have previously found that an incoherent MF blocked 50-Hz MF-induced SAPK activation [25].

Materials and Methods

Antibodies and chemicals

The following chemicals and antibodies were used in the experiment: epidermal growth factor (EGF) (Calbiochem, Darmstadt, Germany), anti-EGFR antibody (Santa Cruz, CA, USA), anti-p-EGFR (Tyr 1173) antibody (Santa Cruz, CA, USA), Nonidet P 40 (NP-40) (Fluka, Buchs SG, Switzerland), propidium iodide (Sigma, MO, USA), Minimum Essential Medium (MEM) (Gibco BRL, NY, USA), mouse anti-goat IgG-FITC (fluorescein isothiocyanate) (Santa Cruz, CA, USA), mouse anti-goat IgG-HRP (horseradish peroxidase) (Santa Cruz, CA, USA)

Magnetic field exposure system

The same sinusoidal MF exposure system used in our previous experiments [26] was used in this experiment. It consists of three groups of square copper coils (36×36 cm^2) placed inside an incubator (Model 3164, Forma, Marietta, GA, USA). The upper, middle, and lower coils (that consist of 168, 60, 168 turns, respectively) are connected in series and spaced 8 cm apart from each other. The coils are placed in an iron metal container with many ventilation holes to shield cells from stray MF. A 50-Hz sinusoidal magnetic field was generated by feeding a line current to the coils. Two power regulators were used to control the flux density. The flux density within the coils can be set from 0 to 0.8 mT. Magnetic flux densities were measured using an EFA-300 EM Field Analyzer (Narda Safety Test Solutions, GmbH, Pfullingen, Germany). When energized, a very uniform magnetic field can be generated within (10×10×10 cm^3) the coils, where cell culture dishes were placed.

Fig. 1. MF signal waveform oscillographs. The upper, middle and lower waveforms show the 50-Hz sinusoidal MF, incoherent ('noise') MF and combined MF, respectively.

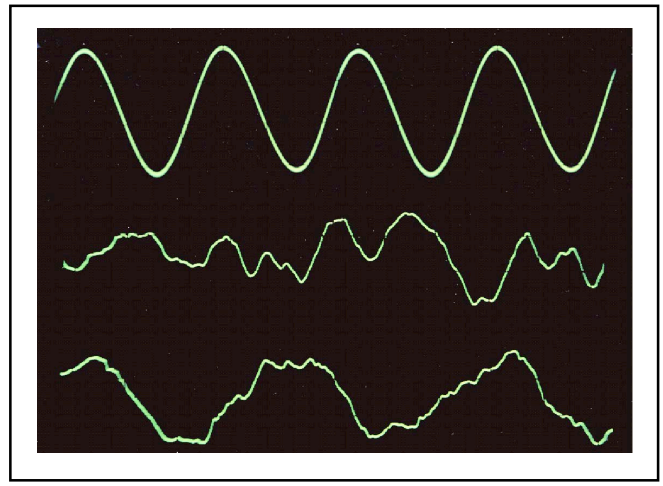
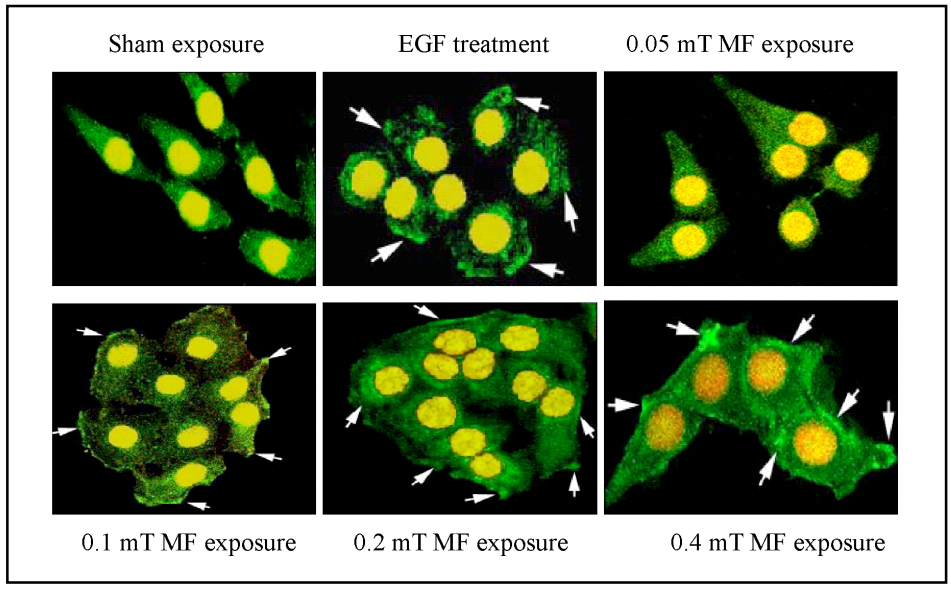


Fig. 2. Effects of 50-Hz MF exposure on EGF receptor clustering. FL cells were exposed to a 50-Hz MF at different intensities for 15 min. EGF treatment served as a positive control. EGF receptor clustering was analyzed using immunofluorescence with confocal microscopy (x 1,000). The arrows indicate EGF receptor clustering.



For incoherent and combined (sinusoidal and incoherent) MF exposure, cells were exposed in another identical incubator, in which three groups of square coils (36×36 cm^2) are double-wrapped with two lines of copper wire. A '30-90 Hz' white-noise signal (provided by Dr. Miguel Penafiel, Catholic University of America, Washington, DC, USA) was sent to one of the double-wrapped wires after magnifying through a power amplifier (CROWN 1400CSL, Crown International Inc., Elkhart, IN, USA). The other copper wire coil was fed by a sinusoidal 50-Hz current during combined MF exposure. The amplitude of the sinusoidal and 'noise' MF could be adjusted to produce a magnetic field strength (rms) according to the experimental requirement.

For sham exposure, the condition was the same as exposure but without current or signal input. Temperature in all the incubators was monitored with a thermocouple probe (sensitivity ± 0.1 °C) attached to the plates near the dishes. During exposure, all incubators were aired with humidified 95% air and 5% CO_2, and all the dishes were kept at 37.0 ± 0.2 °C throughout the entire experiment. The exposure system was turned on at least 2 hrs before an experiment. After exposure, dishes were removed from the chambers while the magnetic field was still on. The waveforms of different MFs were monitored by an oscilloscope (Fig.1). The alternating current (AC) (50 Hz) background field in the incubators was 1–2 μT and the total static MF was 18.5 μT with a 14.1 μT horizontal and 12.0 μT vertical components.

Cell culture and treatments

The experiment included two methods of cell culture, one was used in confocal microscopy analysis and the other in phosphorylation measurement. In confocal analysis, human amniotic (FL) cells were cultured on a glass cover slip placed in a culture dish. Cells used for phosphorylation measurement were cultured in dish directly. Cells were cultured in MEM (Minimum Essential Medium) medium containing 10% fetal calf serum (FCS, Sijiqing Biotech, Hangzhou, China), 100 U/ml of penicillin, 100 μg/ml streptomycin, 100 μg/ml kanamycin, at 37 ± 0.2 °C in humidified 95% air and 5% CO_2. After 48 hrs, FL cells were divided into five groups for the following treatments: a) positive control, cells were treated with 100ng/ml EGF for 15 min, b) sham exposure, c) 50-Hz MF exposure, cells were exposed

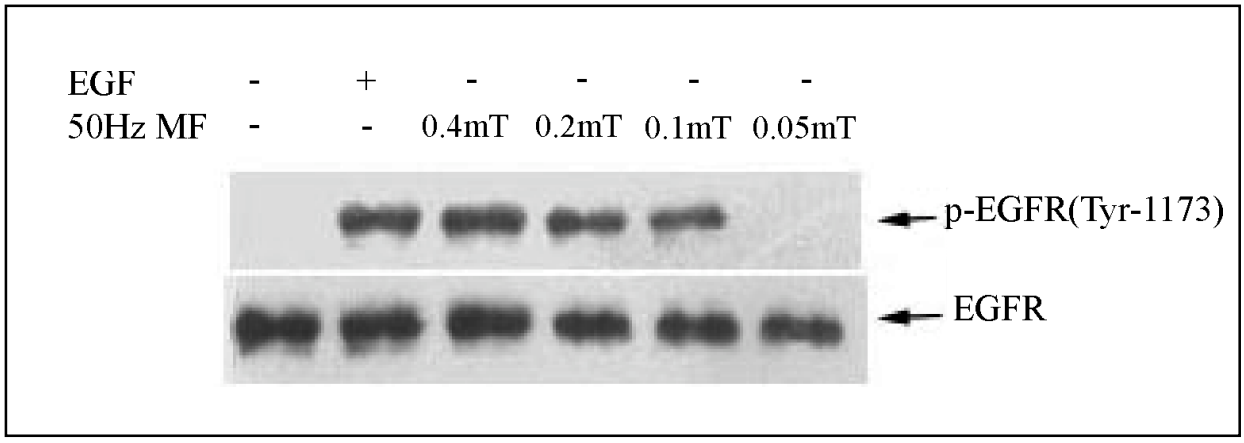


Fig. 3. Effects of 50-Hz MF exposure on phosporylation of Tyr-1173 on EGF receptor. FL cells were exposed to a 50-Hz MF at different intensities for 15 min. EGF treatment served as a positive control. EGF receptor (EGFR) and phosphorylated EGF receptor (p-EGFR) on Tyr-1173 residue were detected using corresponding antibodies with western blot.

to a 50-Hz sinusoidal MF at 0.05, 0.1, 0.2, or 0.4 mT, d) incoherent MF exposure, cells were exposed to 0.2 mT 'noise' MF, and e) combined MF exposure (superposition of 'noise' and 50-Hz sinusoidal MF), cells were exposed to a 0.2 mT 'noise' MF superimposed on a 50-Hz sinusoidal MF at 0.1, 0.2 or 0.4 mT. Exposure-group cells were transferred to corresponding exposure system and exposed for 15 min at the same culture condition. Five dishes were exposed in an exposure system at the same time. The MF was perpendicular to the dishes. All subgroups were harvested at the same time.

Confocal microscopy analysis

Following treatment, cells on a glass cover slip were rinsed with 0.1 M ice-cold phosphate buffered saline (PBS), fixed with 4% paraformaldehyde for 15 min, treated with 0.2% NP-40 for 15 min, sealed with 10% goat serum for 2 hrs, incubated with antibodies of EGF receptor for 1.5 hrs and mouse anti-goat IgG-FITC for 30 min, and then dyed with propidium iodide for 5 min. After every step, cells were rinsed mildly with PBS for 3-5 times. Finally, the cover slips attached cells were sealed on glass slide. Clustering of EGF receptors was analyzed with a confocal microscopy (Leica, TCS-SP Heidelburg, Germany). Experiment was repeated for at least three times.

Phosphorylation measurement of EGF receptor

Following treatment, cells were washed with ice-cold PBS and then lysed in a lysing buffer containing 20 mM HEPES, 2 mM $MgCl_2$, 1% Triton X-100, 1 mM sodium fluoride (NaF), 1 mM sodium orthovanadate (Na_3VO_4), 1μM leupeptin, and 1mM phenylmethylsulfonyl fluoride (PMSF). Cellular protein was extracted and concentration determined with the Bradford's assay. The protein sample was mixed with loading buffer and boiled for 5 min, then separated on a 12% SDS-polyacrylamide gel and subsequently transferred to nitrocellulose membrane for western blotting. EGF receptor and phosphorylated EGF receptor on Tyr-1173 residue were detected using the corresponding antibodies. The blot was developed with enhancement chemiluminescence (ECL). Experiment was repeated for at least two times.

Results

Exposure to 50-Hz MF induced EGF receptor clustering and phosphorylation

Results of immunofluorescence analysis with confocal microscopy showed that treatment with EGF induced receptor clustering in cultured FL cells. Similar to EGF, a 50-Hz sinusoidal MF at 0.1, 0.2 and 0.4 mT also could induce EGF receptor clustering after exposure for 15 min, whereas exposure to a field at 0.05 mT for 15 min did not cause obviously clustering of EGF receptors (Fig. 2). Furthermore, except at 0.05 mT intensity, exposure to a 50-Hz MF at 0.1, 0.2, and 0.4 mT for 15 min enhanced EGF receptor phosphorylation on Tyr-1173 residue (Fig.3).

Incoherent MF inhibited EGF receptor clustering and phosphorylation induced by 50-Hz MF

Unlike 50-Hz MF, results presented in Figs.4 and 5 showed that exposure to a 'noise'-MF alone at 0.2 mT did not induce EGF receptor clustering and phosphorylation on Tyr-1173 residue. Superimposition of the 'noise'-MF completely inhibited EGF receptor

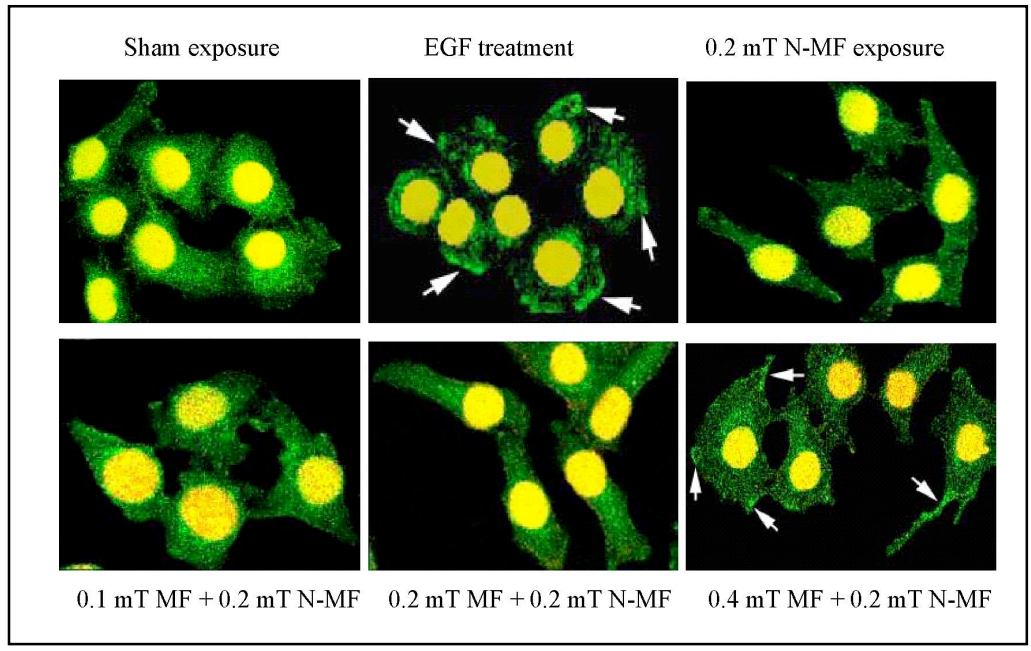


Fig. 4. Effect of superimposing of a 'noise' MF on 50-Hz MF-induced EGF receptor clustering. FL cells were exposed to combined MF for 15 min. A 0.2 mT 'noise' MF was superimposed on a 50-Hz MF at 0.1, 0.2, or 0.4 mT. EGF treatment served as a positive control. EGF receptor clustering was analyzed using immunofluorescence with confocal microscopy (x 1,000). The arrows indicate EGF receptor clustering. N-MF stands for noise-MF.

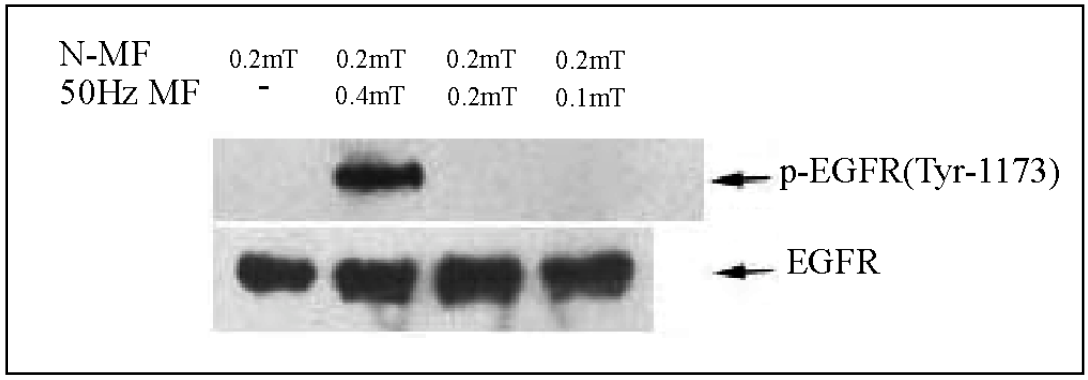


Fig. 5. Effect of superimposing of a 'noise' MF on 50-Hz MF-induced phosporylation of Tyr-1173 on EGF receptor. FL cells were exposed to combined MF for 15 min. A 0.2 mT 'noise' MF was superimposed on a 50-Hz MF at 0.1, 0.2, or 0.4 mT. EGF treatment served as a positive control. EGF receptor (EGFR) and phosphorylated EGF receptor (p-EGFR) on Tyr-1173 residue were detected using corresponding antibodies with western blot. N-MF stands for noise MF.

clustering and phosphorylation induced by 50-Hz MF at 0.1 and 0.2 mT. However, the 'noise'-MF only partially inhibited receptor clustering and phosphorylation induced by a 50-Hz MF of 0.4 mT.

Discussion

Receptor on cellular membrane surface is one of the important interaction sites for extracellular signals, such as hormones, cytokines, etc, and the specific interaction between extracellular signals and their receptors is usually the initiation step for signal transduction. Normally, when a ligand (such as EGF) binds to its corresponding receptor, it will first induce receptor clustering, and then activate the cellular cascade signal transduction pathway. Thus, receptor clustering is usually the initial process of cell signal transduction, and can serve as an index for extracellular stimulus-receptor interaction. Some studies showed that ELF-EMF could activate signal transduction pathways which are usually related with receptors [13-16]. Yet, the initial site of ELF-EMF interaction with cell is unclear. Devary et al. [27] found that ultraviolet (UV) light may activate the SAPK pathway through cellular membrane, and Rosette and Karin [28] confirmed that growth factor and cytokine receptors are the sites from which UV light activates the SAPK cascade. These data are taken as proofs that

receptor could become the target site for physical factors. In previous studies, we found that exposure to a 50-Hz MF at 0.4 mT could induce EGF and TNF receptor clustering in Chinese hamster lung fibroblast cells [22]. Results of the present study showed that exposure to a 50-Hz MF with intensity more than 0.1 mT also could induce the EGF receptor clustering in human FL cells. Like UV, MF could interact with receptors on the cellular membrane. It is well known that the classical initial process of EGF signal transduction is that binding of EGF to the receptor activates a tyrosine-specific protein kinase activity intrinsic to the EGF receptor through phosphorylation of specific tyrosine acceptor sites on the EGF receptor [29]. The carboxy terminal of tyrosine-1173 residue on the EGF receptor is a major site of autophosphorylation, which occurs as a result of EGF-EGF receptor binding [30-32]. Once activated, EGF receptor mediates the binding of the phosphotyrosine binding (PTB) domain of Grb2 and Shc through indirect interactions with Tyr-1173 in the Ras signaling pathway [30, 31]. Tyr-1173 of EGF receptor also functions as a kinase substrate, as demonstrated by the direct phosphorylation by Src to further control cellular proliferation [32]. So the phosphorylation of Tyr-1173 on EGF receptor is an important evidence that indicates whether EGF receptor is activated. Results of the present experiment showed that, except at 0.05 mT, exposure to a 50-Hz sinusoidal MF at 0.1, 0.2 and 0.4 mT could enhance EGF receptor phosphorylation of Tyr-1173. EGF receptor phosphorylation is consistent with its clustering induced by 50-Hz MF. Thus, it was confirmed further that 50-Hz MF not only affected receptor clustering, but also induced receptor activation. The data suggest that receptors on cellular membrane is one of the initial target sites for ELF-MF, which could transfer and transduce its signal into cells. And based on the experiment, we found the threshold intensity of 50-Hz MF on EGF receptor is between 0.05 and 0.1 mT. In previous studies, we found that exposure to a 50-Hz MF could activate SAPK, p38 MAPK and Ras in CHL cells [15, 16, 26]. However, the relationship between activation of SAPK, p38 MAPK, Ras and receptors induced by MF need to be investigated further.

Although there exist controversies, the 'temporal and spatial coherence hypothesis' of EMF has hitherto been supported by many in vitro and in vivo studies [33-39]. Based on their studies, Litovitz et al. [23, 24, 40] indicated that a reasonable hypotheses for accounting for thermal-noise fields being ignored by cells or cells can distinguish between external EMF and thermal noise, is that they do exhibit a distinct difference in spatial behaviors. Because the external electromagnetic 'noise'-fields are temporally incoherent in time, they fail to satisfy temporal coherent criterion and cannot induce biological effects. However, since externally imposed electromagnetic 'noise' is spatially coherent, cells are unable to discriminate against it, and so it is capable of confusing the biological EMF detection mechanism and inhibiting the biological effects induced by applied EMF. According to this hypothesis, it also supposes that receptors on the membrane are the possible biological targets of external electromagnetic fields. A significant number of receptors can be simultaneously activated by exogenous magnetic fields, but not by endogenous thermal-noise fields and exogenous 'noise' magnetic fields. In the present experiments, we found that a 50- Hz MF at intensity more than 0.1 mT could induce EGF receptor clustering on the cellular membrane and enhanced the phosphorylation on Tyr-1173, while the 'noise' MF at 0.2 mT alone could not. When superimposing the 'noise' MF, the biological effects induced by a 50-Hz MF at 0.1 and 0.2 mT were completely inhibited. However, the effects caused by a 50-Hz MF at 0.4 mT could not be completely eliminated by the 'noise' field. The results support the hypothesis that when the effects of coherent MF were eliminated completely by an 'noise' MF, the intensity of the superimposed incoherent-noise MF should not be weaker than the coherent MF. Litovitz et al [23] presumed that the plausible mechanism of a coherent EMF interacting with cells is that the field affects the binding of ligands to receptors and consequently modifies the transduction of the extracellular signal into cells. But, our results showed that without ligand, a 50-Hz MF alone also could induce clustering and phosphorylation of receptors. Thus, this suggests the possibility that 50-Hz and 'noise' MF directly affect the function of cell membrane receptors. Although the actual mechanism needs to be explored further, results from the present experiment support the 'temporal and spatial coherence hypothesis' on possible interaction at receptor pathway level. The hypothesis may provide a new approach to prevent the deleterious effects of ELF-EMF.

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