Effects of extremely low frequency magnetic fields on gap junctional intercellular communication and its mechanism*

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Abstract The study on biological effect of electromagnetic fields has been paid close attention in recent years. Gap junctional intercellular communication (GJIC) plays an important role in the maintenance of cell proliferation and differentiation, and in the multistage process of carcinogenesis. A series of researches showed that extremely low frequency (ELF) magnetic fields not only enhance the inhibition of GJIC induced by 12-*O*-tetradecanoylphorbol-13-acetate, but also inhibit GJIC directly when the intensity is equal to or more than 0.4 mT, and that the mechanisms of GJIC inhibition by ELF magnetic fields are due to hyperphosphorylation of connexin 43, which is mediated by protein kinase C-activated signal transduction, and the internalization of connexin 43 from plasma membrane to cytoplasm.

Keywords: extremely low frequency magnetic fields, gap junctional intercellular communication, mechanism.

Over the past two decades, extensive epidemiological surveys and animal experiments have been conducted to evaluate the biological effects of extremely low frequency (ELF) magnetic fields (MFs) on carcinogenesis. The results have suggested that increased risk for several cancers such as brain tumor, leukemia, and breast cancer, could be associated with exposure to ELF MFs. Some biochemical molecules and pro-oncogenes, such as ornithine decarboxylase (ODC), melatonin, *c-fos*, and *c-myc*, have also been studied[1-3]. However, there still exist a lot of arguments concerning the influence of ELF MFs on cancer development.

Intercellular communication is the basis for homeostasis. Gap junctions are membrane channels that permit the transfer of ions and small molecules between contiguous cells. They are thought to play an important role in the maintenance of cell proliferation and differentiation. Most tumor cells have no or a reduced capacity for gap junctional intercellular communication (GJIC), but when connexin genes are transfected into such cells, normal cell growth is reconstructed and GJIC often recovered. It had also been observed that GJIC could be inhibited by various types of tumor promoters. Therefore, in the multistage process of carcinogenesis, inhibition of GJIC is considered to be an important event during the promotion stage, and GJIC is regarded as an important index of screening possible cancer promoter[4,5]. A series of experiments concerning the effect of 50 Hz MFs on GJIC and its mechanisms were conducted in our laboratory to explore if the ELF MFs may inhibit GJIC acting as cancer promoter or be synergetic with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in cancer promotion, and to explore the molecular mechanism of GJIC suppression by ELF MFs.

1 Effects of 50 Hz magnetic fields on GJIC

The system used to expose cultured cells to 50 Hz ELF MFs consisted of three groups of square copper coils with 36 cm width within a CO_2 incubator. A very uniform MF in the center of the coils could be regulated from 0 to 1 mT. Disks containing cells were placed coaxially with the central line of the coils, and the MFs were perpendicular to the dishes[6]. The GJIC of cells was determined by dye transfer assay with two different methods. One was microinjection. Lucifer yellow was injected with glass capillaries into the cells by negative pulse current. Five minutes later, the number of dye-coupled cells (DCC) per injection of the dye was used as an index of GJIC. The other technique was fluorescence recovery after photobleaching (FRAP) analysis, which was performed with a laser-scanning confocal microscope. The cells were photobleached to 20% ~ 30% of their original fluorescence intensity. They were then examined for the recovery of fluorescence, and the percentage of fluorescence recovery was used as the index of GJIC

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function. Two cell lines, Chinese hamster lung cells (CHL) and mouse fibroblast cell line NIH 3T3, were used in the experiments.

The results with microjection showed that the suppression of GJIC due to ELF MFs exposure was related to the field strength and exposure time (Table 1). After exposure of CHL cells to 0.8 mT MF for 24 h, the number of DCC decreased as compared with control group ($P < 0.05$). Exposure at 0.2 mT or 0.4 mT for 24 h did not suppress GJIC in CHL cells. However, when combined with 5 ng/mL TPA treatment during the last one hour, the number of DCC was significantly lower than the values of the group treated with 5 ng/mL TPA alone[6]. Further investigation showed that 5 minutes of ELF MFs (0.8 mT) exposure had no effect on GJIC, but one hour of MFs exposure significantly inhibited GJIC, and the GJIC suppression induced by 24 hours of MFs exposure was the most significant among the above three levels of exposure duration[7].

The results of FRAP analysis, which is more sensitive and accurate than microinjection, confirmed the above results and further revealed that 0.4 mT, or higher than 0.4 mT ELF MFs could inhibit GJIC, and the threshold of GJIC inhibition induced by ELF MFs alone was 0.4 mT (Table 1).

Table 1. The effects of ELF and/or TPA on GJIC

Treatment	DCC($\bar{x} \pm s$)	FRAP($\bar{x} \pm s$)
Control	$9.84 \pm 2.01(61)$	$41.04 \pm 13.10(45)$
0.2 mT	$9.56 \pm 2.20(25)$	$38.31 \pm 7.23(93)$
0.4 mT	$9.24 \pm 1.39(24)$	$25.50 \pm 9.26(47)^{c)}$
0.8 mT	$6.08 \pm 1.59(24)^{a)}$	$18.58 \pm 7.73(60)^{c), d)}$
TPA	$6.26 \pm 1.39(53)$	$24.21 \pm 8.74(59)$
0.1 mT + TPA	—	$23.79 \pm 8.78(45)$
0.2 mT + TPA	$5.52 \pm 1.53(25)^{b)}$	$12.69 \pm 6.34(68)^{e)}$

a) *vs.* Control, $P<0.05$; b) *vs.* TPA, $P<0.05$; c) *vs.* control, $P<0.01$; d) *vs.* 0.4 mT, $P<0.01$; e) *vs.* TPA, $P<0.01$. In brackets, the number of cells tested. — not measured.

Pulsed electromagnetic fields (PEMFs) are successfully used in treatment of patients with ununited fractures and wound healing. It is supposed that the PEMFs may suppress GJIC to benefit cell proliferation, which is probably one of the important mechanisms for wound healing by PEMFs. The effects of PEMFs with 50 Hz repetition frequency, and 2 ms pulse width on GJIC in CHL cells were studied with microinjection method. The results showed that the GJIC of the cells exposed to the PEMFs at 0.4 mT or 0.8 mT for 24 h were significantly suppressed, and there was significant difference in dye transfer when compared to sinusoidal MFs with the same average flux density, indicating that the PEMFs are more effective than the sinusoidal MFs in down-regulation of the GJIC[8].

It is necessary to ascertain whether the ELF MFs or the induced electric fields (EFs) will cause the inhibition of GJIC. An experiment was designed to compare the effects of exposing the cells to different induced EFs with the same magnetic flux density level. The cells near the center of dishes and 3 cm apart from the center of dishes were examined for GJIC following exposure to sinusoidal MFs at 0.8 mT for 24 h. The results showed that there was no significant difference in dye transfer between the two induced EFs[7]. This confirmed that the inhibition of GJIC was mainly caused by direct action of MF rather than by induced EFs.

Since ability of penetrating MFs in human tissues and cultured cells is similar, the above experiments, together with other researches, led to the conclusion that magnetic fields might act as cancer promoter or work in synergy with other cancer promoters. International Committee of Non-Ionization Radiation Protection (ICNIRP) suggested 0.5 mT as the reference standard of professional exposure to 50 Hz magnetic field[9]. We suggest that the ELF MF exposure standard should be lower based on our experimental results.

2 Mechanisms of ELF inhibiting GJIC

Being exposed to 50 Hz of magnetic field, connexin 43, a structural and functional protein of gap junction, was studied at its transcriptional, translational, and post-translational levels in our laboratory. The exposure parameters were fixed at 0.8 mT and 24 hours. Transcription of connexin 43 gene of the exposed cells was examined by Northern blot analysis using a ^{32}P-labeled connexin 43 probe. The ratio of the quantity of connexin 43 mRNA to glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA was calculated as the relative level of connexin 43 gene transcription. Statistical analysis showed that there was no effect of ELF and/or TPA on connexin 43 gene transcription[10].

The changes in connexin 43 quantity and its phosphorylation after treatment of NIH 3T3 cells for

24 h at 50 Hz and 0.8 mT, with or without treatment of 3 ng/ml TPA for 2 h, were examined by Western blot analysis. The results showed that the ELF MFs and/or TPA exposure induced a decrease in non-phosphorylated connexin 43 (P_0). Meanwhile, cells treated with the ELF MFs and/or TPA displayed a hyperphosphorylated connexin 43 band (P_3). Similar changes of P_0 and P_3 were observed in membrane fraction or in the total protein, and the results showed that Cx43 protein level did not appear to be substantially altered by any of the treatments[18]. Connexin 43 hyperphosphorylation can induce the change of its topology and/or inhibit the assembly of connexin 43. The former can lead to the closure of gap junction channel, and the latter reduce the number of gap junction on membrane. We concluded that hyperphosphorylation of connexin 43 is one of the important mechanisms of GJIC inhibition induced by ELF MFs.

Gap junctions are membrane channels, and the number and location of connexin are important for normal GJIC function. The location of connexin 43 proteins in CHL cells was analyzed by indirect immunofluorescence histochemistry with confocal microscopy. The results showed that after being exposed to 50 Hz, 0.8 mT ELF MFs for 24 h, the CHL cells demonstrated a reduction in plasma membrane-associated connexin 43 immunostaining, and an increase in the cytoplasm, especially near/on the outer membrane of nucleus. Western blot analysis further confirmed that the quantity of connexin 43 protein in cytoplasm increased after treatment of ELF MFs. These phenomena suggested that connexin 43 internalization from plasma membrane to cytoplasm should be related to GJIC suppression induced by ELF MFs.

In the above experiments, TPA, a typical cancer promoter and activator of protein kinase C (PKC), was used as a positive control. There are many similarities between the effects of ELF and TPA, i.e. (i) GJIC can be inhibited by both ELF and TPA in a dose-dependent manner. The inhibition of GJIC by 50 Hz, 0.8 mT, 24 h ELF MFs exposure in CHL or NIH 3T3 cells matches to that of 3~5 ng/mL TPA; (ii) the suppression of GJIC induced by ELF or TPA can be inhibited by PKC inhibitors; (iii) ELF or TPA treatment of cultured cells do not change the level of connexin 43 mRNA; (iv) both TPA and ELF can induce the hyperphosphorylation of connexin 43, and its internalization; and (v) ELF can work in synergy with TPA. However, there are also some differences between MFs and TPA. The inhibition of GJIC induced by ELF MFs is time-dependent, and connexin 43 hyperphosphorylation state is kept on during ELF MFs exposure. But there are special events involved in the GJIC suppression induced by TPA. GJIC inhibition was observed within 2 hours by TPA treatment and then the GJIC was gradually recovered. The connexin 43 hyperphosphorylation was closely related to the change in GJIC, therefore, the hyperphosphorylation of connexin 43 could be induced by different pathways under different stimulation.

3 Relationship between GJIC inhibition and signal transduction

As a weak signal or energy, ELF MFs possibly affect the structure and function of certain proteins located on plasma membrane, then leads to the activation of certain signal transduction pathway and finally affects the function of gap junction. It has been reported that ELF MFs exposure activates PKC and induces PKC gene transcription[3]. To explore if PKC is involved in the connexin 43 hyperphosphorylation, an experiment with PKC inhibitor staurosporine (STS) or palmitoyl carnitina (PMC) was carried out in our laboratory. CHL cells were exposed to 50 Hz, 0.8 mT MFs for 24 h together with the treatment of PKC inhibitor (STS or PMC) at different concentrations during the last one hour. The results showed that the suppression of GJIC induced by ELF MFs was significantly inhibited in the presence of 10 nmol/L STS or 10 μmol/L PMC[11], suggesting that ELF MFs can activate PKC, which leads to the hyperphosphorylation of connexin 43.

A study was also designed with the method of mRNA differential display to screen and identify differentially expressed gene following 50 Hz MFs exposure to Daudi cells in our laboratory. Thirteen fragments responding to ELF exposure were found. Two of them, MF-CA and MF-CB, were demonstrated to be involved in the signal transduction[12,13].

Some oncogene products, as the signal transduction factors, play an important role in the process of GJIC inhibition. GJIC can be inhibited when cells were transfected by oncogenes. It had been discovered that ELF could alter the transcriptional activity of *c-fos* and *c-jun*, and Fos and Jun combining do-

mains were also found in Cx43 promoter sequence[14]. We found that 0.8 mT or 0.4 mT ELF could enhance the transcriptional activity of *c-fos* by TPA[15].

The above findings suggest that signal transduction pathways should be involved in the inhibition of GJIC induced by ELF MFs.

4 Prospect

As a new possible human carcinogen[16], the biological effect of ELF MFs has attracted great attention worldwide. It is believed that MFs achieves its effects on carcinogenesis process by signal transduction pathways. However, the knowledge of ELF on signal transduction is limited, and many links between them should be further elucidated. It is also an interesting question that whether physical factors, such as MFs, and chemical factors, such as TPA, share some similar signal transduction pathways. When chemical factors react with proteins, DNA or other chemicals in cells, physical factors may act as ligands to specifical receptors and achieve their effects, but there is little knowledge about this kind of receptors so far.

Varieties of chemical binding domains on DNA molecules have been discoverd. Are there any binding domains of physical factors exist? Lin et al.[17] reported that a magnetic field domain exists in the promoter sequence of hsp70 gene. Although further study should be done, physical factors, as an information or energy, have their targets on cells. Therefore, it is believed that the reaction of organism to physical factors will be elucidated, and a thorough knowledge of this process will have a great impact on life science.

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