Involvement of MAP Kinase Cascade in EMF Induced Signal Transduction Pathways

by

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Abstract

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We have reported previously that differentiation of HL-60 human leukemia cells from a nonphagocytic suspension culture to an attached fibroblast-like culture with high phagocytic activity elicited by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) could be induced by exposure to a 60 Hz, 1 G electromagnetic field (EMF). In this study, the impacts of EMF on MAP kinase pathway that is know to be involved in TPA induction were investigated in this cell line. We found that a small but significant increase in the level of activation of two members of MAPK kinase (MAPK) cascade, Raf-1 and MAP kinase, could be seen in these cells after they were exposed to the field for a short time (less than 30 minutes). Similar effects of EMF

were also registered in MCF-7 human breast cancer cells and rat fibroblast 3Y1 cells. The levels of Raf-1 and MAPK activation in EMF exposed cells are equivalent to those observed in cells treated with low concentration of TPA (0.05-1 ng/ml). The involvement of PKC in the process leading to activation of MAPK cascade in cells exposed to EMF is also implicated by the present results. MAPK activation is inhibited by an inhibitor to PKC α, but not PKC δ inhibitors, in cells subjected to EMF exposure or TPA treatment. Thus, similarities between the effects of EMF and TPA treatment are supported by this investigation. This provides a possible method for revealing other participants in EMF-cell interaction, since the TPA induction pathway is well documented.

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CHAPTER I

INTRODUCTION

Overview

Exposure to electromagnetic fields (EMF) resulting from environmental proximity to high voltage power lines and electric stations and the routine use of household appliances and cellular phones has increased exponentially in the last half century. This has occurred with the growth of the generation and consumption of electric power and the advent of electromagnetic communication systems. The possible adverse health effects associated with exposure to these low energy electromagnetic fields have been a public concern since an epidemiological study in 1979 showed a high incidence of malignant diseases in children living near 60 Hz high voltage power lines (Wertheimer and Leeper, 1979).

To address this concern, extensive investigations have been conducted in the last two decades involving scientists from several different disciplines. Evidence has accumulated gradually from epidemiological studies that suggest a correlation between EMF exposure and human cancer. The presence of extenuating environmental factors, however, makes it difficult to establish accurately a cause and effect relationship of EMF exposure and cancer. The evidence has been both inconsistent and controversial. In the meantime, various biological effects of EMF exposure were reported in laboratory studies with animal models (*in vivo*) and cell cultures (*in vitro*), though again not without contention.

One of the main reasons that results in the controversy on the effects of EMF is a lack of understanding of mechanisms by which the impact of EMF on biological systems is realized. A number of models have been suggested and vehemently debated. Recent *in vitro* studies seem to support the view that signal transduction pathways may mediate the EMF-cellular interactions. The present research is designed to provide relevant information for determining a possible relationship existing between cell signaling and EMF exposure.

Classification of Electromagnetic Fields

Electromagnetic fields are classified, in increasing order of both frequency and energy, into the following categories (Figure 1): 1) extremely low frequency electromagnetic fields (ELF-EMF), produced by power lines, electrical wiring and electrical equipment; 2) radio and TV broadcast waves (VLF- very low frequency and VHF- very high frequency); 3) microwaves; 4) infrared radiation; 5) visible light, and 6) ultraviolet, x-rays and gamma rays (ionizing radiation). In addition, an electric or magnetic field with a steady and constant strength (0 Hz) is called a "static" electric or magnetic field. Some electromagnetic fields have steep waveforms that do not vary in a regular, sinusoidal manner; they are called "pulsing" electromagnetic fields (PEMF).

The strength of a magnetic field is expressed by two different units, tesla (T) and gauss (G). 1 tesla equals 10,000 gauss. For example, the earth's geomagnetic field is a

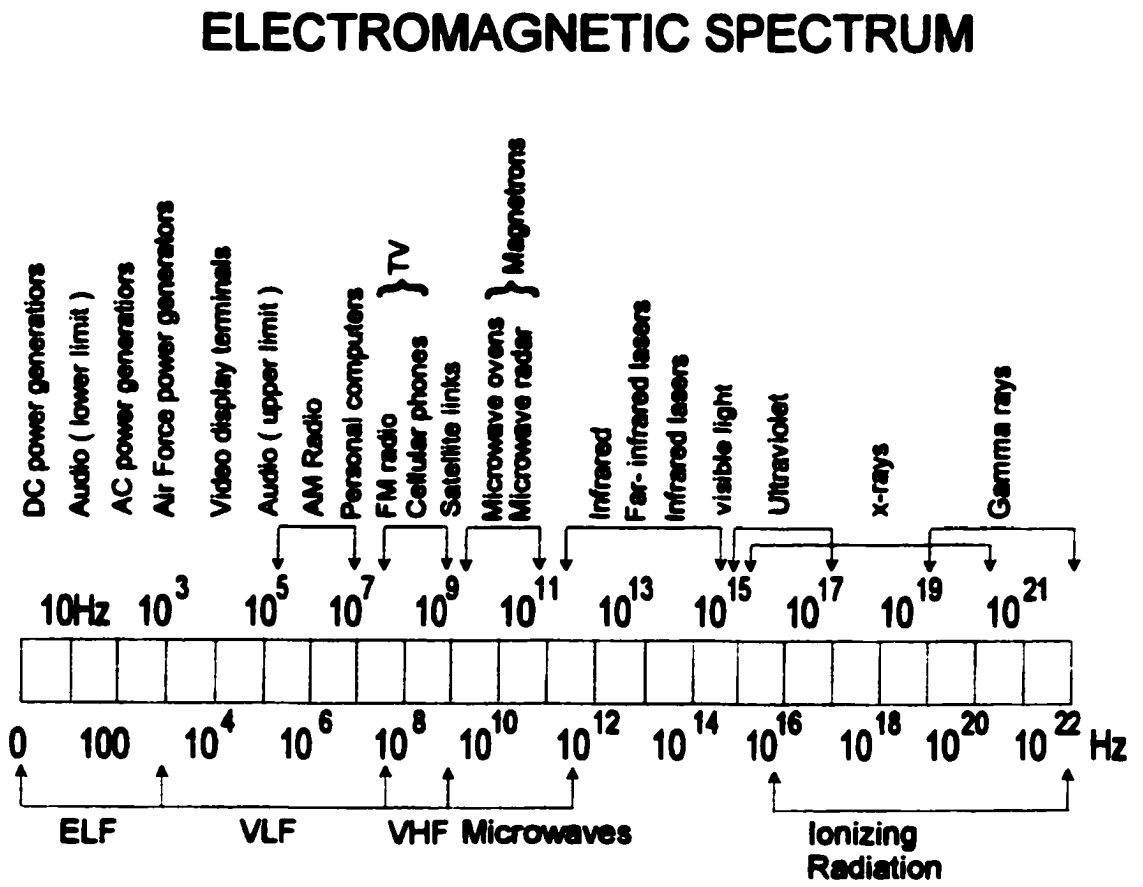


Figure 1: **Spectrum of Electromagnetic Fields.** Examples of typical field strengths from various power sources. X-ray, visible light, microwaves, radio waves, and electric and magnetic fields (EM fields) are all forms of electromagnetic energy. EM fields are generated by power lines, electrical wiring, and electrical equipment. Electric fields are produced by voltage; magnetic fields result from the flow of current through wires or electrical devices. Electric fields are shielded or weakened by materials that conduct electricity. Magnetic fields pass through most materials and are difficult to shield. Both electric and magnetic fields decrease as the distance from the source increases.

static magnetic field of about 50 μT or 500 mG. The strength of an electric field is expressed by the unit volt (V).

It has been known for some time that exposure to ionizing radiation causes DNA damage leading to carcinogenesis. Whether or not electromagnetic field with much lower energy is a potential health hazard is the problem that has led to controversy. With the increasing ubiquity of cellular phones, public awareness of the possible adverse health effects of EMF used for telecommunication reaches new level. At present, numerous studies are concentrated on the impacts of these electromagnetic fields.

EMF and Public Health — Epidemiological Studies

Widespread discussions of the potential hazardous effects of exposure to EMF were triggered by the 1979 report (Wertheimer and Leeper, 1979) that suggested a correlation between childhood leukemia and proximity to power lines (odds radio >2, 95% CI). Subsequent epidemiological studies have primarily been concerned with the role of EMF in tumorigenesis, but the effects of EMF on the reproductive system and the relationship between EMF exposure and some neurodegenerative diseases have also been investigated.

Cancer. Dozens of studies have been conducted on the possible linkage between cancer in children and residential exposure to ELF-EMF. In most of these studies, the intensity of EMF exposure is estimated based on the distance between the residential dwelling and the external power source and, in some cases, the configuration and current-

carrying capacity of the line (called "wire code"). Historical current load of the line was also taken into consideration in some of these investigations.

In the original study in the U.S., wire code configuration was developed as a surrogate method of estimating long-term exposure to EMF emitted from nearby distribution lines. The coding was classified as high-current and low-current configurations. Cancer mortality was examined from death certificates for residents of Denver, Colorado who died at less than 19 years of age during 1950-73. A total of 344 cases of childhood cancer were identified, and 344 population controls were selected from Denver area birth certificates matched on the basis of birth month and county. Exposure was assessed from the wiring coding of the homes occupied at the time of birth and death. They found that children who died of cancer were more likely to have lived in homes classified as high-current configuration than in homes classified as low-current configurations.

Later studies also favor a positive association between childhood cancer and exposure to EMF. In 1988, Savitz *et al.* conducted a case-control study of residential exposure in the same area studied by Wertheimer and Leeper (1979). A total of 356 appropriate childhood cancer cases and 278 population controls were evaluated. The relative risk for all cancers among children living in the high-current classification was 1.5 (95% CI, 1.0-2.3). A study in Los Angeles County (London et al., 1991) focused strictly on the incidence of childhood leukemia indicated that relative risks tended to increase with the intensity of the electromagnetic fields in a linear fashion. The highest value of 2.2 (95% CI, 1.1-4.3) was found in the highest exposure categories (in a five-level wire code configuration).

However, there are other studies that showed no significant correlation between EMF exposure and cancer in children. Linet *et al.* (1997) conducted a large case-control study (638 cases and 620 control) testing whether childhood acute lymphoblastic leukemia was associated with exposure to power frequency EMF. Field measurements were taken in all homes where cases and control had lived for at least 3.5 of the 5 years before diagnosis. Wire codes were assessed for the 416 residentially stable pairs. No indication of an association between wire code and leukemia was found. Based on magnetic-field measurements, for children exposed to 0.2 μT fields, the relative risk was 1.2 (95% CI, 0.9-1.8) for the unmatched analysis and 1.5 (95% CI, 0.9-2.6) for the matched pairs. They concluded that there was not signification association. For those exposed to 0.3 μT or stronger fields, the unmatched relative risk was 1.7 (1.0-2.9). Others argue that the overall results are in line with a positive association and that the high-exposure data in particular indicate a trend toward a positive, field-strength-dependent association between magnetic fields and leukemia risk.

Taken together, the results of the published studies suggested a correlation between childhood leukemia and exposure to ELF electromagnetic fields. The relative risks reported in most of the studies are in the range of 1.5-3.0 (Repacholi and Greenebaum, 1999). A number of potential confounding factors, including traffic density, pollution from car traffic, socioeconomic status, and age of home have been proposed as possible explanations of the observed association between childhood leukemia and power line-based exposure estimates. Several studies showed that adjustment for these factors had little effect on the results (Savitz *et al.*, 1988; London *et al.*, 1991; Feychting and Ahlbom, 1993).

Possible associations between occupational exposures to ELF-EMF and cancer risk have been evaluated in a number of studies. An increased cancer risk among exposed subjects in electrical occupations such as utility workers in generating stations, power line repairers, electricians, welders, and electric railway locomotive drivers was reported The types of cancer for which elevated risks were found, however, differed from one study to another. Thériault *et al.* (1994) and Floderus *et al.* (1993) observed a significant association between EMF exposure and incidence of leukemia, whereas Savitz and Loomis (1995) and Sahl *et al.* (1993) saw no such association. Correlation between EMF exposure and brain tumors was reported in studies by Savitz and Loomis (1995), Floderus *et al.* (1993), and Thériault *et al.* (1994), but not Sahl *et al.* (1993).

Associations for other types of cancer were also investigated in several studies. An elevated risk of testicular cancer in workers exposed to EMF was found in a study by Stenlund and Floderus (1997). Thériault *et al.* (1994) observed an association between lung cancer and pulsed electromagnetic fields (PEMFs), but a negative association was reported by Savitz and Loomis (1995).

There are also studies that reported an increase in chromosomal aberrations in workers exposed EMF (Nordenson *et al.*, 1988, 2001; Valjus *et al.*, 1993; Jelmert *et al.*, 1994). Nordenson *et al.* (2001) found that chromosomal aberrations in electrified railway engine driver increased fourfold compared with controls, which might be related to routine exposure to EMF (the daily time-average magnetic-field strength in the engine was 2 to 15 μT, with occasional peak values up to over a hundred μT).

Neurologic and Psychiatric Diseases. Epidemiological data are sparse on the relationship between neurologic diseases and EMF exposures. Sobel and Davanipouor (1996) observed a threefold increase in the risk of Alzheimer's disease in association with high level of EMF exposure. Association for amyotrophic lateral sclerosis was also reported in a study by Davanipour et al. (1997).

The psychiatric impact of EMF exposure was reported in several studies. An association between suicide and EMF exposure was reported by Reichmanis *et al.* (1979), Perry *et al.* (1981), and Baris *et al.* (1996), but McDowall (1986) saw no such assoication. As to depression, the results were even more controversial. No association was seen between occupational exposure to EMF and symptoms of depression in electric power transmission and distribution workers (Broadbent *et al.* 1985). However, the studies of Savitz *et al.* (1994) indicated an elevated risk for depression among electricians.

EMF and Public Health II — Clinical Studies

Cardiovascular. The effects of EMF exposure on cardiovascular system were reported in several studies. Small changes in cardiac function were detected in human volunteers exposed to combined 60 Hz electric and magnetic fields (Maresh *et al.*, 1988; Graham *et al.*, 1994; Cook *et al.*, 1992). Graham *et al.* (1994) found that resting heart rates were slightly but significantly reduced (about 3-5 beats/minute) during or immediately after exposure to a 60 Hz electromagnetic field (9 kV/m, 0.02 mT). Exposure to weaker (6 kV/m, 0.01 mT) or stronger (12 kV/m, 0.03 mT) fields, however,

did not have this effect. Studies by Graham *et al.* (1992) showed that while continuous exposure to 60 Hz EMF at 9 kV/m, 0.02 mT reduced the heart rates, intermittent exposure could both reduce and increase the heart rates. These changes in heartbeat were rather small and within normal range. Acute or long-term cardiovascular-related hazards have not been detected at levels below current exposure standards for ELF or radio frequency fields (Jauchem, 1997).

Brain and behavior. The possible interaction of brain and central nervous system with EMF was investigated in several studies. Visual evoked potentials were affected by 50 Hz fields with intensities of 60 mT or more (Silny, 1986), but not by 50 or 60 Hz fields of low intensities (Sander *et al.*, 1982; Graham *et al.*, 1994). Exposure to combined electric and circularly polarized 60 Hz magnetic fields at different strengths (6 kV/m, 0.01 mT; 9 kV/m, 0.02 mT; 12 kV/m, 0.03 mT) was shown to have an impact on the auditory event-related potentials (Cook *et al.*, 1992). There are also studies that reported changes in response latency for complex reasoning tasks in volunteers who had weak power-frequency electric currents passed through electrodes attached to the head and shoulders (Stollery, 1986; Podd *et al.*, 1995).

Hormone and immune system. Only a few studies have been conducted to evaluate the effect of EMF exposure on immune system and reproductive hormones. Jonai *et al.* (1996) found that the cytokine production of human peripheral blood mononuclear cells was affected by 50 Hz electromagnetic fields (1, 3, 10, 30 mT). Other reports (Selmaoui *et al.*, 1996, 1997) showed that acute exposure for one night to a linear polarized magnetic field at 10 μT has no effect on hormonal or immune parameters in healthy male volunteers.

Field-related suppression of the hormone melatonin has been proposed as a possible mechanism for the association between increased cancer risk and exposure to EMF (Stevens, 1987; Stevens and Davis, 1996). Alterations in melatonin production were reported in people sleeping at home under electric blankets (Wilson *et al.*, 1990). Several studies found that occupational exposure to electromagnetic fields reduced the level of melatonin in electric utility workers (Burch *et al.*, 1998) and video display unit workers (Arnetz and Berg, 1996). A decrease in nocturnal excretion of 6-hydroxymelatonin sulfate, the major metabolite of melatonin, in the urine samples of female garment workers (Juutilainen *et al.*, 2000), railway workers (Pfluger and Minder, 1996), and electric utility workers (Burch *et al.*, 1998, 1999) was also reported.

Biological Effects of EMF — Laboratory Studies

Numerous laboratory studies both *in vitro* and *in vivo* show that exposure to EMF induces a wide range of responses in biological systems. The impact of EMF exposure on cell proliferation and differentiation, cell cycle progression, apoptosis, inter-cellular communications, DNA replication and gene expression has been reported in many of these studies. However, it is still unclear how EMF brings about these effects, and how the relationship between the observed biological effects and increased cancer risk associated with EMF exposure can be understood.

Cell proliferation and differentiation. Pulsing electromagnetic fields (PEMF) generated by noncontinuous electric currents have been employed in treatment of ununited bone fractures and failure arthrodeses for two decades. The field stimulated cell proliferation and differentiation may provide explanations of the healing effect of PEMF.

Studies by Fitzsimmons *et al.* (1992) showed that a low-amplitude (10mV/cm), low-frequency (less than 100 Hz) electric field promoted the proliferation of bone cells by inducing the release of insulin-like growth factor II into the culture medium. Similar experiments on rabbit costal chondrocytes suggested that differentiation could be induced by PEMF *in vitro* (Hiraki *et al.*, 1987).

The impact of EMF on proliferation and differentiation of other cell types was reported in several studies. Human fibroblasts were induced to differentiate into postmitotic cells upon exposure to a 20 Hz EMF at 8 mT (Rodemann *et al.*, 1989). An immediate and transient increase in PKA (cyclic AMP-dependent protein kinase) activity in cells exposed to EMF triggered a modulation of the differentiation process (Loschinger *et al.*, 1998; Thumm *et al.*, 1999). Rosenthal and Obe (1989) found that cultivating human peripheral lymphocytes (HPL) in the presence of a 50-Hz EMF leaded to enhancement of cell proliferation *in vitro*. This is supported by a recent *in vitro* study indicating that proliferation of T-cells, which compose 70-80% of the lymphocytes in peripheral blood, was affected by a complex 1.8 mT pulsed EMF (Electrobiology, Inc.), a 0.1 mT, 60 Hz power frequency EMF, and a 0.2 mT, 100 Hz sinusoidal EMF (Johnson *et al.*, 2001). In contrast, in another study, Zwingelberg *et al.* (1993) showed that exposure of rats to a 50-Hz, 30-mT magnetic field had no effect on proliferation characteristics of peripheral lymphocytes. Induction of differentiation by EMF exposure in cultured hematopoietic progenitor cells was reported by Tao and Henderson (1999). They found that differentiation of HL-60 cells from a promyeocytic form (hematopoietic progenitor cells) to phagocytic marrophages was induced by a 60 Hz, 1G electromagnetic field. The effect of EMF on differentiation is approximately equivalent to treatment of cells with the

tumor promoter 12-0-tetradecanoylphorbal-13-acetate (TPA) at the concentration of 250-500 pg/ml, and the effect of EMF exposure and TPA treatment is additive at low TPA concentration.

Cell cycle progression and apoptosis. The impact of EMF on cell cycle progression and apoptosis was evaluated in several studies. Conti *et al.* (1999) reported that the number of phytohaemaglutinin-activated or non-activated lymphocytes in S phase increased after 48 hours of exposure to ELF-EMF with respect to non-exposure controls. Similar effects were observed in human peripheral blood mononuclear cells (PBMC) exposed to ELF-EMF for 24, 48, or 72 hours (Felaco *et al.*, 1999). A significant increase of percentage expression of cell cycle progression in the S phase was seen in exposed cells compared to non-exposed cells, while in G1 and G2 phases, there were no differences.

Induction of apoptosis in different cell lines exposed to electromagnetic fields was reported in a couple of studies. Flipo *et al.* (1998) found that *in vitro* exposure to a static magnetic field (250-1500G) increased apoptosis in lymphocytes and macrophages. Simko *et al.* (1998) examined the effects of applying ELF-EMF for different durations (24, 48, and 72 h) and different field intensities (0.1-1.0 mT) on micronucleus (MN) formation and induction of apoptosis in a human squamous cell carcinoma cell line (SCL II) and a human amniotic fluid cell line (AFC). A statistically significant increase of MN frequency and induction of apoptosis in SCL II cells was seen after 48 and 72-hour continuous exposure to 50 Hz magnetic field (MF) (0.8 and 1.0 mT). However, exposure of AFC cells to EMF of different intensities and for different exposure times showed no statistically significant differences when compared with controls. Apoptosis was also

induced in rat tendon fibroblast (RTF) and rat bone marrow (RBM) osteoprogenitor cells by exposure to an AC (60 and 1000 Hz, up to 0.25 mT) and a DC magnetic field (up to 0.25 mT) (Blumenthal *et al.*, 1997). However, a new study by Ruiz Gomez *et al.* (2001) with two U-937 (a histiocytic lymphoma) and HCA-2/1cch (a human colon adenocarcinoma) showed that exposure of these two cell lines to a 25 Hz EMF at 1.5 mT had no impact on cell cycle distribution and apoptosis.

DNA replication. The influence of EMF on cell cycle progression is also demonstrated in the effects of EMF on DNA replication. As reported by Liboff *et al.* (1984), human fibroblasts exhibited enhanced DNA synthesis when exposed to sinusoidal magnetic fields of wide range of frequencies (15-1000 Hz) and amplitudes (2.3×10^{-6} to 5.6×10^{-4} Tesla). A mild increase (20%) in DNA synthesis was observed in rat osteosarcoma cells under growth-limiting conditions after 34 hours of exposed to sinusoidal 60-Hz electric currents at intensities of 300-400 mA r.m.s./cm^2 (Noda *et al.*, 1987). An effect of pulsing electromagnetic fields on DNA synthesis was reported by Takahashi *et al.* (1986). They found that DNA synthesis in Chinese hamster V79 cells was significantly enhanced when they were exposed to weak, pulsing electromagnetic fields generated by specific combinations of the pulse width (25 microseconds), frequency (10, 100 Hz) and magnetic intensity (2×10^{-5}, 8×10^{-5} T), whereas the DNA synthesis of cells in the fields at 4×10^{-4} T was repressed to 80% of that in controls. A recent study by Nindl *et al.* (1997) showed that a 1.8 mT, bone healing, electromagnetic field and power frequency EMFs of 0.1 and 0.4 mT significantly inhibit DNA synthesis in otherwise unstimulated Jurkat (E 6.1) cells.

However, it is still controversial as to whether DNA replication is affected by EMF exposure. Zhao *et al.* (1999) indicated that enhanced DNA synthesis seen as higher level of incorporation of [^{3}H] thymidine in magnetic field-exposed C3H 10T1/2 mouse fibroblasts cells (0.1-0.8 mT, 60 Hz) seemed to be attributable at least in part to a slight elevation in temperature during exposure.

Gene expression. Alternation in gene expression induced by EMF exposure is one of the mechanisms that have been used to explain the increased relative risks of cancer following EMF exposure. It was suggested that the effects of ELF-EMF were restricted to a group of normally expressed genes involved in growth and/or differentiation (Goodman and Henderson, 1991).

Early studies with the salivary gland cells of the dipteran, *Sciara coprophilia*, showed that EMF exposure resulted in the activation of RNA transcription as measured by the increased ^{3}H-uridine incorporation (Goodman and Henderson, 1983). The nascent RNA in exposed cells had the predicted S value for mRNA (Goodman *et al.*, 1986). An analysis of the grain count distribution over the X chromosome of the exposed Sciara salivary glands cells in transcription autoradiograms exhibited an increase in radioactive uridine uptake both in sites corresponding to active ones in control cells and also in loci where there were no detectable transcription in unexposed cells (Goodman *et al.*, 1987). Two-dimensional gel analysis of the pattern of protein translation of the exposed cells demonstrated both qualitative and quantitative alterations as compared with the control cells; this pattern was different from that exhibited after heat shock (Goodman and Henderson, 1988).

Later studies revealed that transcription of some of the genes participated in regulation of cell proliferation and differentiation was altered in mammalian cells exposed to EMF. Phillips *et al.* (1992) employed the nuclear run-off assay to assess alterations in specific gene transcription in CEM-CM3 T-lymphoblastoid cells exposed for 15-120 min to a 1 gauss sinusoidal magnetic field at 60 Hz. They observed time-dependent and cell density-dependent changes in the transcription of c-*fos*, c-*jun*, c-*myc* and PKC α. The impact of EMF exposure on the expression of early response genes c-*fos*, c-*jun*, and c-*myc* was also reported in several other studies. Lagroye and Poncy (1998) showed that c-*jun* c-*fos* genes were induced in primary rat tracheal epithelial cells and two related immortalized cell lines by a magnetic fields (50 Hz, 100 microT[rms] sinusoidal magnetic field combined with a 55 microT geomagnetic-like field) with patterns and amplitudes similar to those seen when cells were exposed to ionizing radiation. It was suggested in a studies with rat pheochromocytoma PC12 cells that magnetic fields increased c-*fos* expression only in cultures that were not yet exhibiting maximal c-*fos* expression (Campbell-Beachler *et al.*, 1998). The responsive sites to EMF exposure in c-*fos*, c-*myc*, and HSP70 were defined. The upstream regulating region of c-*fos* from –363 to –225, which includes the SRE/AP-1 sites, a 900 base pair segment of the c-*myc* promoter, containing eight nCTCTn sequences, and a 70 bp region of the HSP70 promoter, containing three nCTCTn sequences were required for the induction of c-*fos*, c-*myc*, and HSP70 expression by electromagnetic fields, respectively (Rao and Henderson, 1996; Lin *et al.*, 2001). The influence of EMF exposure on cytokine production in peripheral blood mononuclear cells was investigated by Pessina and Aldinucci (1998). They found that a 50-Hz pulsed electromagnetic field significantly

increased the level of interleukin-1beta and tumor necrosis factor alpha in cells stimulated with phytohemagglutinin, leading to significant increase in proliferation indexes.

Melatonin. The impacts of EMF exposure on pineal and serum melatonin levels in mammals have been reported in *in vivo* studies. As early as 1981, Wilson *et al.* found that exposure to a 60 Hz electric field significantly suppressed the normal nocturnal increase in pineal melatonin content in rats. Later studies showed that the nocturnal peak of serum melatonin concentration was also depressed in rats exposed to power frequency EMF (Loscher *et al.*, 1994; Selmaoui and Touitou, 1995; Mevissen *et al.*, 1996), and the studies indicated that magnetic fields altered the production of melatonin through an inhibition of pineal N-acetyltransferase activity (Selmaoui and Touitou, 1995). Investigations with isolated pineal glands of Djungarian hamsters (Brendel *et al.*, 2000) and primary pinealocyte cultures of rodents (Rosen *et al.*, 1998) demonstrated that the suppression of melatonin synthesis observed in animals exposed to EMF was caused by direct effects of the fields on pineal gland.

These reports support the speculation of suppression of melatonin by EMF as a possible explanation for the linkage between residential exposure to EMF and increased cancer risk (Stevens, 1987; Stevens and Davis, 1996), since melatonin has been shown to be able to slow down the growth of certain types of cancer cells, including breast cancer cells (Blask and Hill, 1986).

In vitro studies with cell lines, on the other hand, revealed that in addition to affecting melatonin production, EMF exposure might also exert influence on melatonin cellular interaction. Liburdy *et al.* (1993b) found that growth inhibition of melatonin on human estrogen-responsive MCF-7 breast cancer cells was blocked by exposure to a 60

Hz, 12 mG sinusoidal magnetic field when melatonin was present at physiological concentrations (1 nM), though the field alone had no effect on MCF-7 proliferation in the absent of melatonin. Similar effects of 12 mG field on inhibition of MCF-7 cell growth by melatonin were reported in later investigations (Harland *et al.*, 1997; Blackman *et al.*, 2001).

The underlying mechanism of the effects of EMF on antiproliferative action of melatonin was explored in several laboratories. Ubeda *et al.* (1995) showed that melatonin-induced enhancement of junctional transfer in normal C3H/10T1/2 cells was blocked by exposure to a 50 Hz magnetic field. It is indicated in a study by Ishido *et al.* (2001) that EMF caused uncoupling of signal transduction from melatonin receptors to adenylyl cyclase in MCF-7 cells. Melatonin-induced inhibition of cAMP accumulation was eliminated by exposure to a 50 Hz electromagnetic field at 1.2 and 100 microT for 3, 5 and 7 days in a time-dependent manner. [125I]melatonin binding assay and RT-PCR analysis demonstrated the involvement of melatonin 1a receptors. These investigations provide evidence that EMF may affect the growth of cells by disrupting intercellular communications as well as by acting at cellular level involving receptors and signal transduction pathways.

Tumor promotion and co-promotion. Unlike ionizing radiation and other environmental carcinogens, electromagnetic fields of much lower frequency do not possess adequate energy to cause direct changes in chromosomal structure or DNA sequences. Up to now, there is no evidence that such fields could induce the gene mutations that initiate the cancer process. They may, however, play a role in tumor promotion and /or co-promotion in the multi-step and multifactorial carcinogenesis

process. By altering the delicate balance in the biological system, EMF may potentiate the malignant environment, promoting cancerous activity in cells that are already precancerous.

This hypothesis was tested in animal models and cell lines. Skin tumor development in mice, a well-accepted, convenient model for the study of multistage carcinogenesis was employed in a couple of investigations. In a study by Mclean *et al.* (1991), the dorsal skin of juvenile female mice were treated with a subthreshold dose of the carcinogen7,12-dimethyl-benz(a)anthracene (DMBA), then a week after the treatment, the mice were sham exposed or field exposed at 60 Hz, 2 mT 6 h/day for 21 weeks to test whether EMF would act as a tumor promoter. No papilloma was developed in these mice. The possible role of EMF as a co-promoter was also explored in this study. TPA was applied weekly to mice pre-treated with a sub-carcinogenic dose of DMBA. These mice were then exposed or sham exposed to the field. Tumors developed earlier in mice exposed to both EMF and TPA, but the difference in the time of onset was not statistically significant. Rannug *et al.* (1993) conducted similar experiments with a 50 Hz field (50 μT and 0.5 mT) to test the possibility that EMF acts as a tumor promoter. They saw no difference in skin tumor formation between control and EMF exposed groups, however they observed a slight increase in leukemia among animals exposed to a 0.5 mT field, though it was not statistically significant. The impact of radio frequency (RF) electromagnetic fields on mice predisposed to develop cancers was investigated in several studies. Long-term intermittent exposure to pulsed 900 MHZ electromagnetic fields was shown to increase the incidence of lymphoma among E mu-Pim1 transgenic mice that carry a lymphomagenic oncogene (Repacholi *et al.*, 1997). However, chronic,

low-level exposure of mice prone to mammary tumors to 435 MHz radio frequency (RF) radiation did not affect the incidence of mammary tumors, tumor growth rate, latency to tumor onset or animal longevity when compared to sham-exposed controls (Toler *et al.*, 1997).

More direct evidence that EMF play a role in tumor co-promotion is provided by *in vitro* studies with C3H/10T1/2 murine fibroblasts. An enhancement in neoplastic transformation was only seen in C3H/10T1/2 cells exposed 24 h to 2.45 GHz microwaves at 4.4 W/kg preceded or followed by X irradiation, then subjected to treatment with TPA at a concentration of 0.1 μg/ml (Balcer-Kubik and Harrison, 1991). Without TPA, EMF alone had no effect on neoplastic transformation of the cells. This cooperation between EMF and TPA in promoting cancer was indicated in a later report as well. Cain *et al.* (1993) found that the presence of a 60 Hz, 1 G field increased the TPA-induced focus formation of the transformed UV-TDTx10e fibroblast cells co-cultured with parental C3H/10T1/2 cells, though the field had no effect on focus formation by itself. It suggested that EMF might participate in disrupting communication between normal and transformed cells, which was assumed to be involved in tumor promotion, and influence membrane related events since EMF helped TPA relieve the suppression imposed by C3H/10T1/2 cells on the growth and focus formation of UV-TDTx10e cells. The possible effects of EMF on gap junction communication implicated in this study were further investigated by Li *et al.* (1999). Their research provided direct evidence that 24 h exposure to EMF (50 Hz, 0.8 mT field), like treatment with TPA (5 ng/ml), resulted in a decrease in gap junction communication between Chinese hamster lung cells. It also

demonstrated that combined treatment with both EMF and TPA had a significantly stronger effect than TPA alone.

Overall, it seems that the experimental data to date do not give strong support to the suggestion that EMF exposure has the ability to promote malignant progression alone. A role in tumor co-promotion with known tumor promoters is more likely for electromagnetic fields in light of the current state of investigations.

Mechanism of EMF-cell interaction — EMF and cell signaling

The extremely low energies associated with low-frequency electromagnetic fields, usually much smaller than the thermal noise level, make it very hard to explain how such fields are capable of producing the reported various bioeffects. However, a number of studies in the past two decades indicated that EMF-cellular interaction might be achieved via the mediation of signal transduction pathways (Figure 2).

Cell surface receptors and ion channels. There are speculations that the initial cellular responses to EMF exposure, as to other extracellular stimulations, were generated at the cell surface. This hypothesis has the backing of an early experiment by Marron *et al.* (1988) that showed an augmented negative charge and a reduced hydrophobic character on the cell surface induced by the fields. Later investigations indicated that cell surface constituents such as membrane-receptor complexes and ion-transporting channels were affected by EMF as well. Redistribution of cell surface receptors (transferrin receptors and low density lipoprotein receptors) was observed in cells subjected to

externally applied AC electric fields in the 3 to 23 V/cm range (Cho *et al.*, 1994). A study published recently reported that tyrosine phosphorylation of T cell receptor (TcR) complex was initiated in human T cell line Jurkat exposed to ELF-EMF of 0.1 mT (Lindstrom *et al.*, 2001). 5 minutes of field exposure activated the Src kinase p56lck and resulted in tyrosine phosphorylation of the zeta chains in Jurkat cells, which underlines the possibility that EMF induces signaling sequences similar to those seen upon TcR activation, or even activated TcR *per se*, since tyrosine phosphorylation of the zeta chains happens immediately after the activation of TcR.

A role for ion-transporting channels in transducting EMF signals across the cellular membrane has long been suspected. The Ca^{2+} channel was implicated in a couple of experiments as a plausible site of field interaction (Liburdy, 1992; Kim *et al.*, 1998). Galvanovskis and Sandblom (1997) proposed that stochastic systems such as ion channels provided a basis for signal amplification, and could therefore, despite the low signal-to-noise ratio of the primary response to EMF , lead to propagation of the weak signals along the cell signaling pathways. They presented a theory, based on the formalism of stochastically driven processes, that relates the time averages of the ion channel currents to the amplitude and frequency of the applied signal. According to this theory, the signal-to-noise ratio increased with the number of channels, the magnitude of the rate constants, and the frequency response of the intracellular sensing system (for instance, a calcium oscillator). The amplification properties of an ion channel model were deduced from numerical simulations. Based on numerical estimation of the parameters, they demonstrated that under optimum conditions, even very weak low-frequency electromagnetic signals (<100 Hz and down to 100 μT) might be detected in a cellular

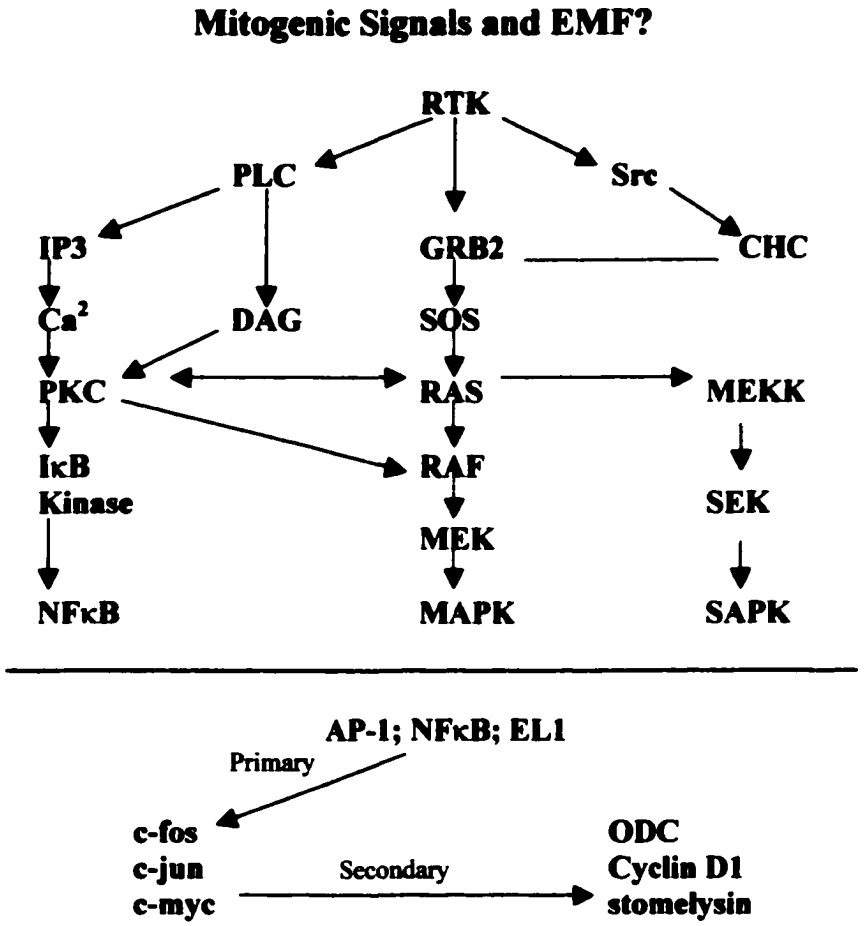


Figure 2: **Proposed pathways that mediate cell-EMF interaction.**

system with a large number of ion channels.

Protein tyrosine kinases. The participation of protein tyrosine kinases (PTKs), prominent members of cell signaling system, in cellular response to EMF stimulation has been explored in a series of studies. Uckun *et al.* (1995) discovered that the Src protooncogene family PTK LYN as well as its downstream substrate SYK, another PTK, was activated in B-lineage lymphoid cells exposed to a low energy electromagnetic field (60 Hz, 1 G). This led to tyrosine phosphorylation of multiple substrates. Subsequent investigations with DT40 lymphoma B-cells showed that, in addition to LYN and SYK, the Bruton's tyrosine kinase (BTK), a member of the Src-related TEC family of PTKs acting downstream of LYN kinase, was also stimulated by EMF exposure (Dibirdik *et al.*, 1998; Kristupaitis *et al.*, 1998). Src kinase p56lck was implicated in EMF elicited signal transduction in Jurkat cells when Lindstrom *et al.* (1995a) found that CD45 phosphatase, which regulates the tyrosine kinase activity of p56lck by removing an inhibitory phosphate, was prerequisite for EMF induced oscillations of free intracellular calcium. This was confirmed by the latest report shown that EMF exposure activated p56lck in Jurkat cells (Lindstrom *et al.*, 2001).

Lipid signal transduction. The impacts of EMF on lipid signaling were registered in experiments with human hematopoietic cell line TF1. Clejan *et al.* (1995) found that exposure to EMF decreased erythropoietin-stimulated phosphatidylinositol 3-kinase activity to lower than basal levels as well as preventing membrane translocation of the 85-kDa regulatory subunit (p85) of the kinase. In addition, they demonstrated that phosphatidylinositol-specific phospholipase C was also activated in TF1 cells, as reflected by increases in diacylglycerol (DAG) and inositol trisphosphate 15-60 seconds

after EMF exposure. Fields induced changes in lipid second messengers were further explored in a later study (Clejan *et al.*, 1996). The results from this investigation suggested that EMF exposure caused up-regulation of phospholipase D (PC-PLD) pathway, as indicated by increased level of DAG, and temporary inactivation of the phopholipase C (PC-PLC) pathway in TF1 cells.

There is evidence that PTKs may be involved in the lipid signal transduction elicited by EMF. Exposure of DT40 lymphoma B cells to ELF-EMF has been shown to result in an increase of inositol-1, 4, 5-trisphosphate levels that relied on the activity of phopholipase Cγ2 (PLC-γ2) (its Src homology domain 2 essential for producing this response) (Dibirdik *et al.*, 1998). Since the proper function of LYN, SYK and BTK was essential for the activation of PLC-γ2 by EMF exposure (Dibirdik *et al.*, 1998; Kristupaitis *et al.*, 1998), the results suggested that LYN-regulated stimulation of SYK and BTK, which act downstream of LYN and upstream of PLC-γ2, played a crucial role in mediating EMF-induced increase in inositol phospholipid turnover.

Calcium signaling. As a ubiquitous response to many stimuli initiated at the cell membrane, Ca^{2+} flux leads to activation of several important signal transducers such as protein kinase C (PKC). The influence of EMF on intracellular Ca^{2+} oscillations have been reported in a couple of studies. Welleczek and Liburdy (1990) found that rat thymic lymphocytes activated with the mitogen Concanavalin A (Con A) exhibited a 2.7 fold increase in $^{45}Ca^{2+}$ uptake when co-stimulated 60 minutes by exposure to a 60 Hz sinusoidal electromagnetic field. Subsequent research revealed that the responsiveness of the lymphocytes to EMF exposure depended upon the status of the cells and intensity of the field (Walleczek and Budinger, 1992). Experiments with Jurkat cells showed that

oscillations of free $[Ca^{2+}]i$ could be affected by ELF-EMF in a manner similar to that seen when cells were subjected to stimulation by antibodies (Lindstrom *et al.*, 1993, 1995b).

The central role of calcium signaling in EMF-induced cellular responses was underlined in studies indicating that Ca^{2+} influx was essential to the induction of immediate early genes such as c-*myc*, c-*fos etc.* by the fields. Lymphocytes that failed to exhibit increased calcium influx in response to magnetic fields plus Con-A also failed to exhibit an increase in c-*myc* transcription (Liburdy *et al.*, 1993a). Extracellular Ca^{2+} reduction and depletion negated the increased steady state transcript levels of both c-*myc* and c-*fos* observed in HL-60 cells exposed to a 60 Hz EMF (Karabakhtsian *et al.*, 1994). These results strongly suggested that the intracellular second messenger Ca^{2+} was involved in propagating the changes initiated by EMF at the cell membrane down the signal transduction cascade into the nucleus.

Protein kinase C. There is evidence that PKC might be induced by EMF in a calcium and phospholipid dependent fashion. Monti *et al.* (1991) detected an elevated level of PKC activity in HL-60 cells exposed to EMF that was eliminated by EGTA. PKC was activated in NALM-6 cells exposed to ELF-EMF as a result of the induction of protein tyrosine kinase LYN and SYK (Uckun *et al.*, 1995). Other studies indicated that that EMF could use Ca^{2+} phospholipid-dependent PKC induction in a way similar to that of TPA or other mitogens (Wallaczek, 1992; Goodman *et al.*, 1993). However, a recent report by Tuinstra *et al.* (1998) demonstrated that EMF by itself does not cause PKC activation in HL-60 cells. No enhancement in PKC activity was observed in cells exposed to a 60 Hz, 1.1 mT field alone or to a combination of the field and 2 μM of TPA for 1 h,

while a greater decrease in cytosolic and a larger increase in membrane PKC activity than was induced by either TPA treatment alone or TPA and sham EMF exposure was seen in cells preexposed to a less than optimal concentration of TPA (50 nM) for 45 min, followed by a 15 min exposure to TPA and EMF. This suggested that rather than initiating a new response, EMF is more likely to exert its impact on enhancing or depressing a molecular reaction already in progress.

Potential Role of MAP Kinase Cascade in Mediating EMF-Cell Interaction

Key position of the MAP kinase cascade in signal transduction pathways. MAP kinase cascade is a well-known cytoplasmic kinase cascade that is responsible for transducing extracellular mitogen stimuli to the nucleus in many cell types, leading to the activation of early response genes such as c-*fos* and c-*jun* (Troppmair *et al.*, 1994; Agarwal *et al.* 1995). It consists of three serine/threonine kinases that act sequentially within one pathway. Raf-1 or B-Raf, MAP/ERK kinase1 or 2 (MEK1 or MEK2), and mitogen activated protein kinase or extracellular signal-regulated kinase 1 or 2 (MAPK/ERK1 or 2) constitute the best known mammalian MAP kinase cascade (see Figure 2).

The MAP kinase pathway is induced by extracellular mitogens such as EGF, PDGF, NGF, insulin *etc.* through receptor tyrosine kinases. Ligand binding causes these receptors to autophosphorylate on tyrosine residues; the phosphotyrosine residues of

autophosphorylated receptors then bind the SH2 domains of adapters, such as Grb2 (growth factor receptor-bound protein 2). The adapters recruit guanine nucleotide exchange factors (SOS) with proline-rich SH3 domain-binding sites to the membrane in proximity to the small G proteins they activate. Exchange factors promote the association of Ras with GTP. The GTP-bound form of Ras binds Raf-1 and B-Raf, thereby targeting one or both Raf isoforms to the plasma membrane for activation by tyrosine phosphorylation (Marais *et al.*, 1995). MEK is activated by Raf via serine phophporylation, and is the upstream activator of MAPK (Huang *et al.*, 1993).

As a signal integrating and processing enzyme, Raf has the hallmarks of a critical switch that connects growth factor receptor activation at the cell membrane with transcriptional events in the nucleus (Daum et al., 1994). The members of the Raf protein kinase family are evolutionarily highly conserved, including D-Raf from Drosophila, Ce-Raf from C. elegans, and A-Raf, B-Raf and c-Raf-1 found in vertebrates. All Raf proteins contain three highly conserved regions, CR1, CR2, and CR3 (Figure 3). CR1 has a binding domain for Ras followed by a zinc-finger motif, through which the activity of the membrane-binding Raf-1 could be regulate by Ras (Roy *et al.*, 1997). CR2 contains several serine and threonine residues. The phosphorylation state of some of these residues influences the activity of Raf. CR3 possesses the catalytic domain, while CR1 and CR2 form the regulatory domain of the Raf kinases.

It has been suggested that Ras binding alone could not activate Raf fully without additional factors. The involvement of a Raf kinase kinase that exists in activated receptor tyrosine kinase-protein tyrosine kinase complex is implicated. The membrane translocation of Raf indicates the possible role of a certain lipid cofactor, but this cofactor

has not yet been identified. PKC is another upstream activator of Raf, as made evident by its ability to induce Raf activation via phosphorylation (Kolch *et al.*, 1993). It seems that the activation of Raf by PKC triggers the same signaling pathways as Raf activation by ras in combination with tyrosine phophorylation (Marquardt *et al.*, 1994). There are indications that Raf is regulated at multiple levels by several distinct mechanisms (Dent *et al.*, 1995; Morrison, 1995). For example, targeted mutation of residue Arg89 of Raf-1, which is required for the association of Raf-1 and Ras, prevents Ras-mediated, but not PKC- or tyrosine kinase-mediated activation of Raf-1 in the baculovirus expression system, suggesting that the activity of Raf-1 could be modulated by both Ras-dependent and independent pathways (Morrison, 1995).

Phosphorylation events play a crucial role in regulating Raf-1. The activation of c-Raf-1 is always accompanied by hyperphosphorylation that can be easily recognized by a retardation of the protein in SDS polyacrylamide gel electrophoresis. When serum starved cells are stimulated by insulin or PDGF, c-Raf-1 is phosphorylated mainly on serine residues. Only faint tyrosine and threonine phosphorylation can be detected (Heidecker *et al.*, 1992).

Three major serine phosphorylation loci of c-Raf-1 from PDGF stimulated NIH3T3 cells were identified by Morrison *et al.* (1993). They were Ser43, Ser259, and Ser621. Ser621 is phosphorylated even in unstimulated cells. It is suggested that the phosphorylation is a co-translation event rather than part of c-Raf-1 activation, and the phosphorylation results in the inability of c-Raf-1 towards further activation. The Ser259Ala mutant has a two-fold higher basal activity than wild type c-Raf-1 and

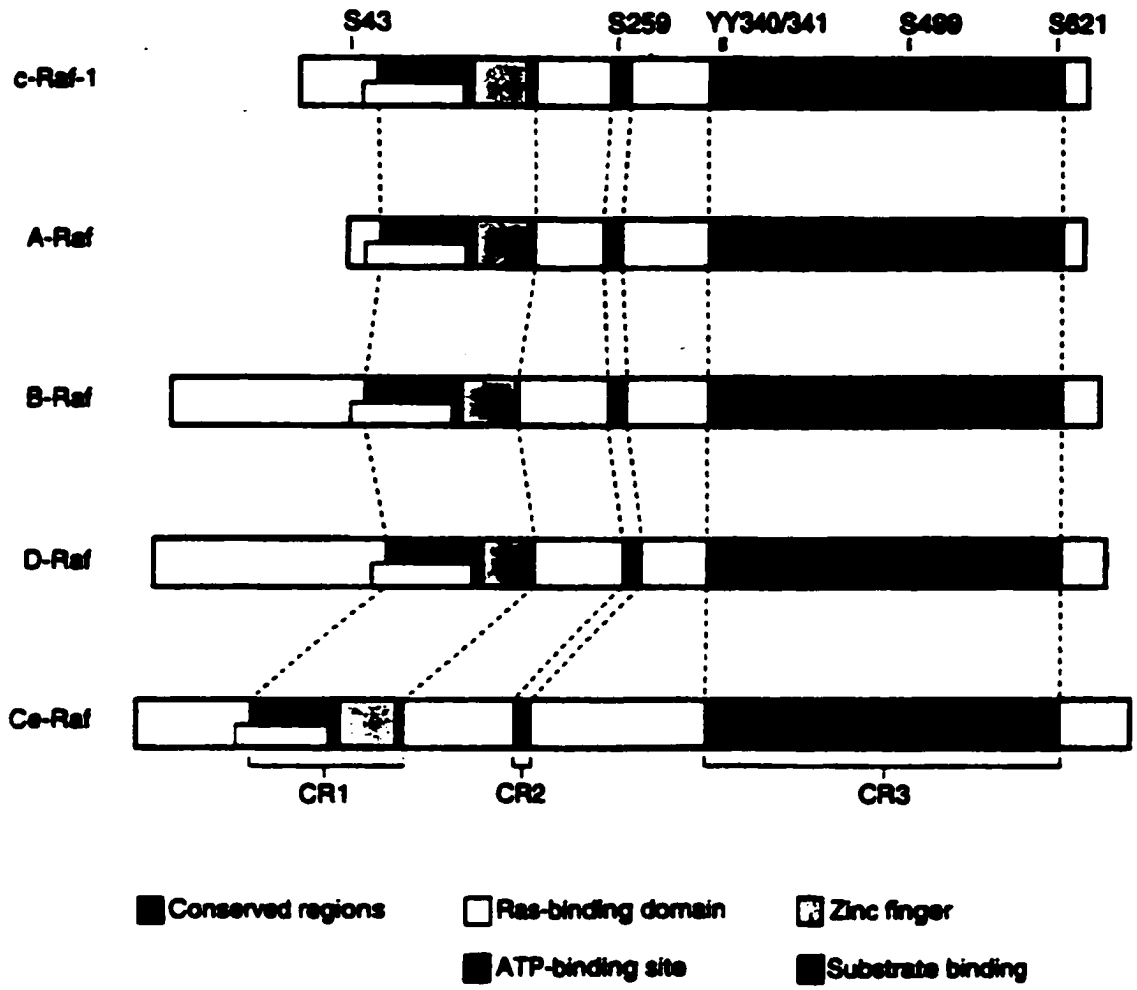


Figure 3. **Structure of Raf kinases.** A-Raf and B-Raf are mammalian isozymes; D-Raf and Ce-Raf are from *Drosophila* and *C. elegans*. CR1 (residues 62-196; numbers refer to c-Raf-1), CR2 (residues 255-268) and CR3 (residues 331-625) represent conserved regions [from Daum *et al.*, 1994].

possesses the capacity to transform NIH3T3 cells. This is probably due to a conformational change resulting in a constitutively activated kinase. Ser43 can be phosphorylated by cyclic-AMP-dependent protein kinase (PKA). The phosphorylation of Ser43 has inhibitory effects on Ras-mediated activation, possibly by impediment of Ras-Raf binding. For c-Raf-1, residues phosphorylated by PKC-α and Src have been identified as well. C-Raf-1 is phosphorylated at Ser 499 or Ser 259 by PKC-α in TPA treated fibroblasts. Ser499Ala mutant has no impact on the basal level of c-Raf-1 activity, but its activation by PKC-α is reduced significantly. However, the response of this mutant to Ras-Lck activation is unaffected (Kolch *et al.*, 1993). It is indicated that PKC-ε, another isozyme of the PKC family, might also stimulate Raf in a Ras-independent pathway.

Src family PTKs are responsible for the phosphorylation of Tyr340 and Tyr341 of c-Raf-1. Phenyalanine substitution of either Tyr340 or Tyr341 produces a protein that could not be activated by Ras-Lck or even by PKC-α *in vitro* (Fabian *et al.*, 1993). Tyrosine phosphorylation of c-Raf-1 was also detected *in vivo*. C-Raf-1 from interleukin treated CTLL-2T cells is highly phosphorylated on tyrosine residues. Also, it appears that tyrosine phosphorylation is correlated with the activity of c-Raf-1, since it can be inactivated by phosphotyrosine specific phosphatase, but not by Serine/Threonine specific phosphatase (Turner *et al.*, 1993). Later studies reported that c-Raf-1 tyrosine phosphorylation could be induced by ionizing radiation in human laryngeal squamous carcinoma cells (PCI-04A) as well (Kasid *et al.*, 1996).

Interestingly, MAPKs such as ERK1 or ERK2 are able to phophorylate c-Raf-1 (Lee *et al.*, 1992; Ueki *et al.*, 1994). A putative function of this feedback phosphorylation

of c-Raf-1 by downstream protein kinases may be related to the dissociation of activated Raf from the plasma membrane.

The hyperphosphorylated active Raf would then stimulate its downstream targets by phosphorylation. MEK1 is phosphorylated at Ser218 and Ser222; either is sufficient for activation (Alessi *et al.*, 1994; Yan and Templeton, 1994). MAPKs, such as EK1 and EK2, are subsequently phophorylated and activated by MEK. The activated MAPKs act upon myriad of downstream effectors, for example c-Fos, c-Jun, and *c*-Myc, causing an alteration in transcription pattern that leads to proliferation, differentiation and other cellular events.

Raf kinase occupies unique position in the intracellular signaling system. It is a critical gate keeper in mitogenic signal transduction and oncogenic transformation, as well as an information integrating center, collecting extracellular stimulations via Ras and PKC and delivering these signals to the nucleus through MAPK cascades; while at the same time, it is a molecular switch, translating tyrosine kinase signaling to serine/threonine phosphorylation and connecting growth factor receptors with transcription factors (Figure 2).

ERK1 and ERK2 were the first of the ERK/MAP kinase subfamily to be cloned. Other well-documented members of the subfamily include: two ERK3 isoforms, ERK4, Jun N-terminal kinases/stress-activated protein kinases (JNK/SAPKs), p38/HOG1, and MAP kinases. Sequence resemblance among the MAP kinases is most apparent in subdomains V, VII, IX, XI, and a long insert between subdomains X and XI. The presence of a regulatory phosphorylation lip (surface loop between subdomains VII and

VIII) with related sequence and the conserved dual phosphorylation sites is another shared feacture of these kinases (Figure 4).

MAP kinase is activated only when both of these two conserved residues, a threonine and a tyrosine, one residue from each other (e.g., Thr183 and Tyr185 in ERK2), are phosphorylated by MEK. *In vivo* and *in vitro*, phosphorylation of tyrosine residue precedes phosphorylation of threonine residue, although phosphorylation of either of them can occur in the absence of the other. Since phosphorylation of both residues is required to activate the MAP kinases, phosphatases that remove phosphate from either site will inactivate the kinases. Certain dual specificity phosphatases selectively inactivate MAP kinases by dephosphorylating both sites (Hunter, 1995).

Biochemical and structural analyses of mutations of the two sites reveal how the activity of the MAP kinases is controlled by the phosphorylation lip (Figure 4). ERK2 mutant T183E has similar structure and basal activity to that of wild type kinase, but a single phosphorylation at Tyr185 elevates its activity to about 100-folds of the basal level, indicating that glutamate partially mimics the negative-charged phosphorylated threonine (Robbins *et al.*, 1993). The crystal structures of three ERK2 mutants at Tyr185 suggest that ERK2 activation leads to conformational changes of the phosphorylation lip (Zhang *et al.*, 1995). In these mutants, 15 residues of the phosphorylation lip from Asp173 to Ala187 are disordered. It appears that Tyr185 is essential in maintaining the low activity conformation, and any change to this site would affect the activity of the enzyme.

The mutation studies of ERK2 also indicate that the phosphorylation lip is not a stable structure, and that conformational modification in this region could be elicited by

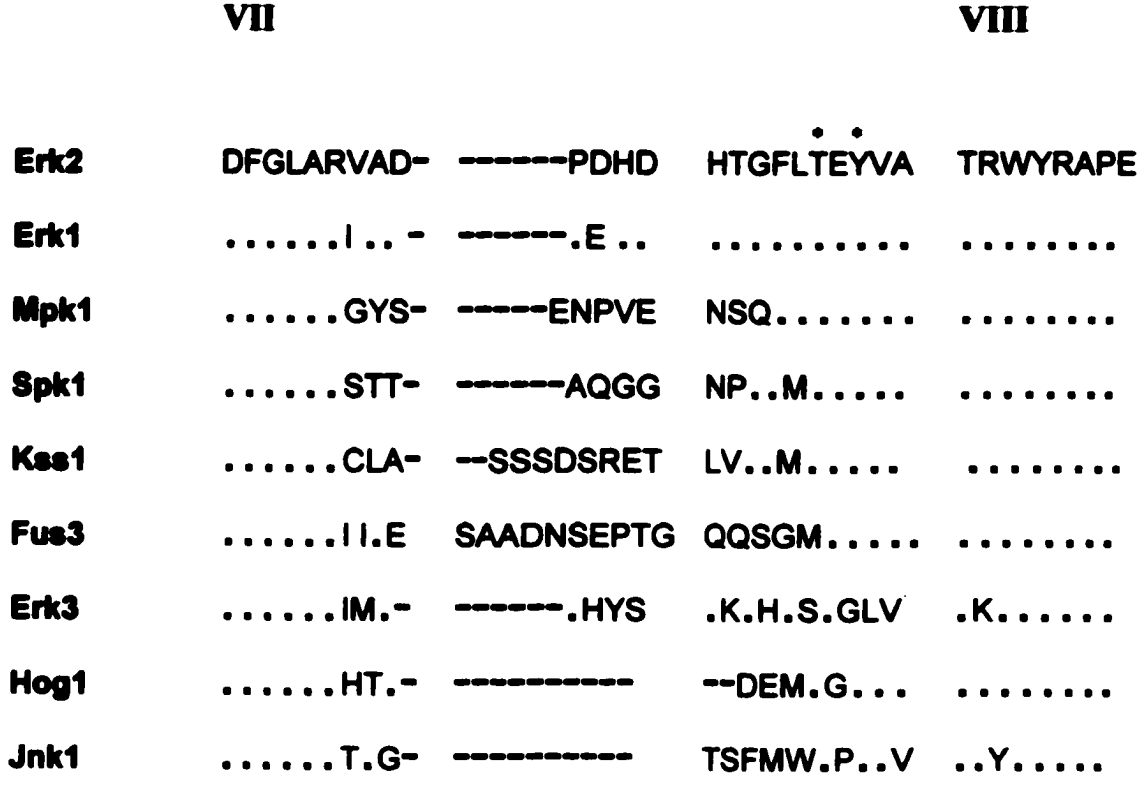


Figure 4. **Alignment of phosphorylation lip sequences of ERK/MAP kinase family members.** ERK1, ERK2, ERK3, HOG1, and JNK1 are mammalian enzymes. MPK1, KSS1, and FUS3 are from budding yeast and SPK1 is from fission yeast. *Dots* indicate identities; *dashes* indicate deletions. The phosphorylation sites are denoted by an *asterisk* [From Cobb *et al.*, 1995)].

modest amounts of binding energy. For phosphorylation of the two residues by MEK to be accomplished, the phosphorylation lip must assume a different conformation and, after phosphorylation, another conformation that is compatible with high catalytic activity. Tyr185 is not easily accessible in the low activity conformation of ERK2, yet it is phosphorylated before Thr183. It seems that the binding of MEK to ERK2 induces a conformational change in the phosphorylation lip that dislodges Tyr185 from its buried position and exposes it to the active site of MEK (Cobb and Goldsmith, 1995).

After activation, MAP kinase translocates into the nucleus where it regulates many of the physiological targets of the MAP kinase signal transduction pathway including transcription factors such as Elk-1, c-Myc, c-Jun, and c-Fos via phosphorylation, leading to transcription activation of early response genes such as c-*myc*, c-*jun*, and c-*fos* (Paris and Pouyssegur, 1991; Ainbider *et al.*, 1997; Leppa *et al.*, 1998). A human MAP kinase isoform p41 MAPK could be found in both cytoplasma and nucleus, and it stimulates the activity of the N-terminal transactivation domain of c-Myc by phosphorylation of c-Myc at Ser62 (Seth *et al.*, 1992). Activation of ERK-1 via Raf-1 and p21ras dependent signals causes hyperphosphorylation of c-Jun (Agarwal *et al.*, 1995). Another member of the ERK/MAP kinase subfamily JNK (Jun N-terminal kinase) binds to and phosphorylates c-Jun at the activating sites of Ser63 and Ser73 (Davis, 1995). These findings support the claim that the MAP kinase pathway represents a significant mechanism of transducing the signal initiated at the cell surface from growth factor-receptor interaction to the nucleus that results in the modification of gene expression.

Potential role of MAPK cascade in mediating EMF-cell interaction. Since MAPK cascade occupies a crucial position in cell signaling system, it is conceivable that

MAPK cascade might be an important participant of the signal sequence elicited by EMF as well. Early investigations provide evidence that upstream activators of MAPK cascade, for example, protein tyrosine kinases (Uckun *et al.*, 1995; Kristupaitis *et al.*, 1998; Dibirdik *et al.*, 1998), PLC-γ2 (Kristupaitis *et al.*, 1998; Dibirdik *et al.*, 1998), PKC (Monti *et al.*, 1991; Tuinstra *et al.*, 1998), and Ca^{2+} (Wallaczek, 1992; Liburdy 1992; Karabakhtsian *et al.*, 1994; Kim *et al.*, 1998), could be induced by EMF, prompting the speculation that MAPK pathway might also be affected by EMF. In addition, steady state transcript level of early response genes (e.g., c-*fos*, c-*myc*, and c-*jun*) was elevated in cells exposed to EMF (Rao and Henderson, 1996; Lagroye and Poncy 1998; Campbell-Beachler *et al.*, 1998; Lin *et al.*, 2001), as what would happen when cells are stimulated with mitogens, suggesting that MAPK cascade might be activated by EMF as well, as transcription activation of the early response is the direct outcome of the induction of MAPK pathway by mitogens.

Previous studies in our lab showed that differentiation of HL-60 cells from a promyelocytic form (hemapoietic progenitor cells) to phagocytic macrophages can be induced by exposure to 60 Hz, 1 Gauss field, as well as by treatment with tumor promoting phorbol ester 12-*0*-tetradecanoylphorbal-13-acetate (Tao and Henderson, 1999). This finding provides a lever for investigating the role of MAPK cascade in EMF-cell interaction, since TPA-induced MAPK pathway has been studied extensively (Adams and Parker, 1991; Kharbanda *et al.*, 1994; Marquardt *et al.*, 1994; El-Shemerly *et al.*, 1997). By comparing the two induction systems, one could expect to gain more insight into the relationship between cell signaling and EMF exposure.

CHAPTER II

MATERIALS & METHODS

Abbreviations

- DTT: dithiothreitol
- EDTA: ethylenediamine tetra-acidic disodium salt
- PBS: phosphate-buffered saline
- PMSF: phenylmethysulfonic fluoride
- SDS: sodium dodecyl sulfate
- TBS: Tris-buffered saline
- TEMED: NNNN-tetramethylethylenediamine
- TPA: 12-tetradecanoylphorbol-13-acetate

Reagents and solutions

- 1X PBS (pH 7.4): 137 mM NaCl; 2.7 mM; 4.3 mM $Na_2HPO_4 \cdot 7H_2O$; 1.4 mM KH_2PO_4
- SDS lysing buffer: 100 mM Tris•HCl (pH8.0); 100 mM NaCl; 20 mM EDTA; 1% SDS
- 1X Stofell Buffer: 10 mM Tris•HCl, pH 8.3; 50 mM KCl
- 1X TBS (pH 7.4): 2.7 mM KCl; 136 mM NaCl; 20 mM Tris-base
- 2X lysing buffer: 20 mM Tris•HCl, pH 7.4; 300 mM NaCl; 3% Triton X-100; 2 mM EDTA; 0.4 mM Na_3VO_4; 0.4 mM PMSF; 2 X protease inhibitor cocktail set I

(1 mM AEBSF, 2 μM E-64, 1 mM EDTA, 2 μM Leupetin, 2 μg/ml Aprotinin, purchased from Calbiochem)

- 2X SDS/sample buffer: 50 mM Tris•HCl, pH 6.8; 20% glycerol (v/v); 4% SDS; 200 mM DTT; 0.2% Bromophenol blue
- 6X SDS/sample buffer: 150 mM Tris-HCl, pH6.8; 36% glycerol (v/v); 10% SDS; 600 mM DTT, 0.6% Bromophenol blue
- 4X Tris•Cl/SDS (pH 8.8): 1500 mM Tris-base, adjust to pH 8.8 with HCl; 4% SDS
- 4X Tris•Cl/SDS (pH 6.8): 500 mM Tris-base, adjust to pH 6.8 with HCl; 4% SDS
- 5X SDS Electrophoresis buffer: 125 mM Tris-base; 960 mM glycine; 5% SDS
- Transfer buffer: 25 mM Tris-base; 192 mM glycine; 20% methanol (v/v)
- Blocking buffer: 5% nonfat milk in 1X PBS or TBS
- Wash buffer: 0.1% Tween 20 in 1X TBS

Cell lines

The HL-60 cell line was derived from a patient with acute promyelocytic leukemia. The subline we use was obtained from Dr. I. Weinstein, Columbia University Health Sciences, New York. The cells were maintained in RPMI 1640 (Gibco BRL life Technology) with 10% fetal bovine serum (Sigma), 1% antibiotic-antimycotic (Gibco). The medium was changed every other day to maintain rapid exponential growth of the

cells. The viability of cells was determined by trypan blue dye exclusion; cell density was determined by hemocytometer.

The human breast cancer cell line MCF-7 was obtained from Dr. R. Goodman, Columbia University Health Sciences, New York. Rat fibroblast 3Y1 cells, 3Y1 cells overexpressing c-Src pro-oncogene, and 3Y1 cells overexpressing v-Src oncogene were obtained from Dr. D. Foster, Hunter College-CUNY, New York. These cell lines were maintained in Dulbecco Modified Eagle Medium (DMEM), purchased from Gibco, containing 10% fetal bovine serum (Sigma) and 1% antibiotic-antimycotic (Gibco).

Exposure Conditions

Cells were exposed to a 60 Hz, 1 G EM field in a Helmholtz Coil Exposure System designed by Electric Research and Management, Inc. (ERM, State College, PA). The exposure conditions were selected as those typical of other experiments that have detected the effects of EMF on signaling pathways. The ERM exposure system provided a graded series of field settings that were maximized by a function generator. A sine-wave generator (Wavetek 11-MHZ function generator, model 21) with variable frequency control was used. The exposure coils consisted of two double-wound coils in an approximate Helmholtz configuration. The coils were supported by an acrylic frame in which the test samples were placed. The coils were placed in 2 mu metal cans, each inside a separated compartment of the same double-door incubator. The ambient magnetic fields in this incubator have been measured over a period of 5 years at about 2

mG. Flasks were placed on a plexiglass stand in the horizontal plane in an area shown to have a uniform magnetic field and maximum field strength inside the coil (Figure 5). Field characteristics were measured using a Tektronix 2245A oscilloscope and a Metex Digital Multimeter.

All experiments were conducted in T-25 culture flasks. Before each experiment, logarithmically growing HL-60 cells (0.8-1.2 × 10^6 cells/ml) were starved in medium containing 0.5 % fetal bovine serum (FBS) for 16-20 hours (Lu *et al.*, 1997). The cell concentration was then adjusted to 1 × 10^6 cells/ml. 15 ml cells were aliquoted into T-25 flasks (control and experimental) one hour before the experiments. MCF-7 and 3Y1 cells were grown in T-25 flasks to near confluence (3Y1 and C-Src 3Y1) or until about 70% of the flask surface was covered (MCF-7 and v-Src 3Y1). They were then starved in low serum medium (0.5%) for 16-20 hours prior to each experiment (Lu *et al.*, 1997). The cells in control and experimental flasks were subcultures of the cells from one single flask to minimize discrepancies between the cultures.

The coils were turned on at least 30 minutes before exposure, and remained in that state while the flasks were being removed to make sure cells were exposed to a stable field. The cells were subjected to: (a) no treatment, (b) EMF exposure, (c) treatment with TPA at different concentrations and (d) EMF exposure imposed upon TPA treatment. All experiments were under the same environmental conditions. TPA was dissolved in DMSO. An equal amount of DMSO was added to the control flasks. The final concentration of DMSO in the medium was 0.1%.

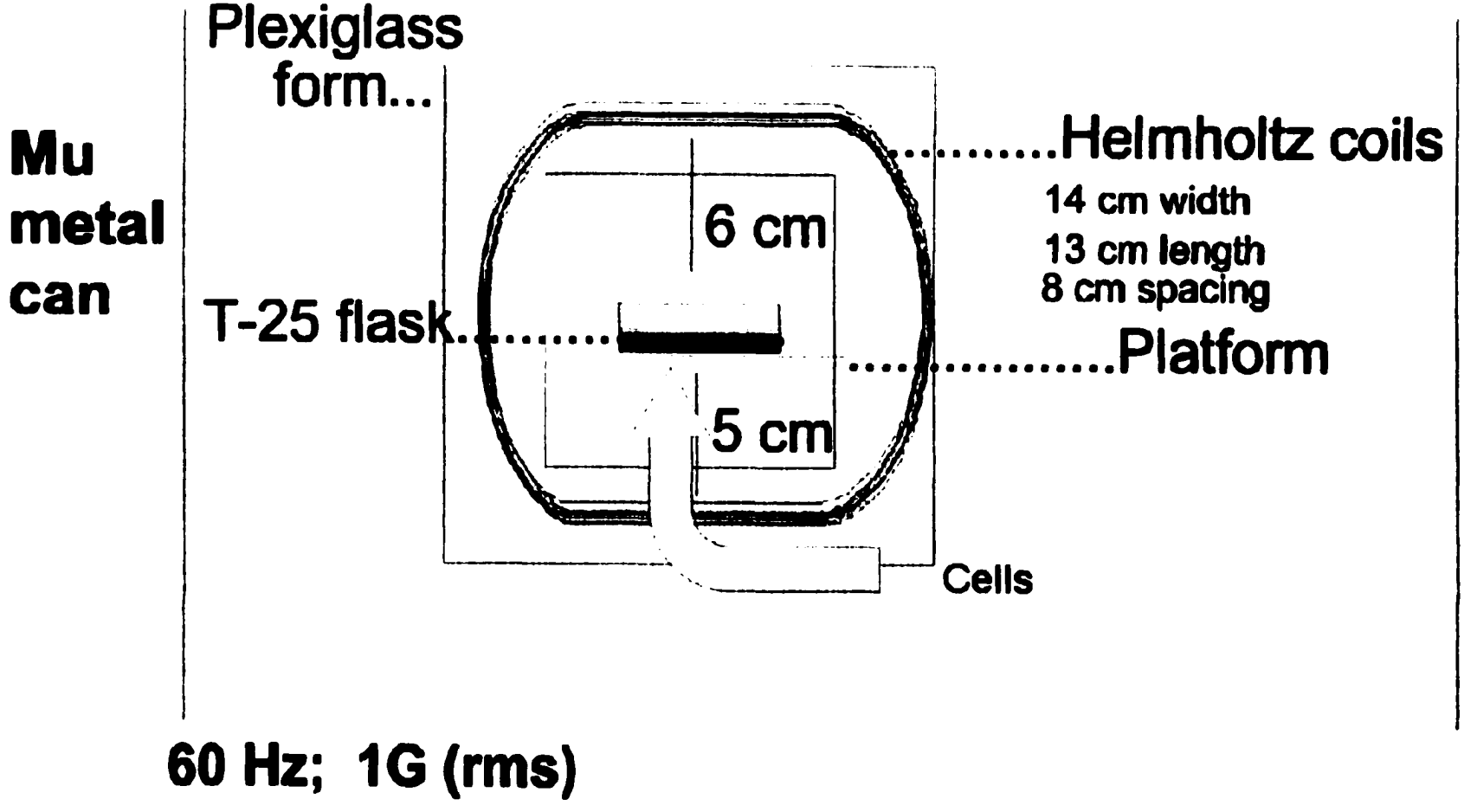


Figure 5. **Exposure conditions.** Temperature inside the mu meatal can is monitored with a Physitemp thermocouple probe sensitive to change of one tenth of a centigrade.

Isolation of RNA

Following exposure, flasks were placed immediately in an ethanol-ice slurry for 10 minutes. Cells were collected by centrifugation at 1,500 rpm for 5 minutes and then washed with ice-cold 1 x PBS. SDS lysing buffer was added and the cells were lysed by forced them though a 20 G needle 15 times. Total cellular RNA was isolated using the phenol-chloroform extraction method with lithium chloride/ethanol precipitation (Maniatis *et al.*, 1989). Contaminating DNA was digested with DNase I for 30 minutes on ice. Several phenol-chloroform extractions were then performed followed by precipitation of RNA in ethanol. Each RNA sample was tested by 1% agarose gel electrophoresis to determine the integrity of RNA based on the 5S, 18S and 28S bands and DNA contamination, following ethidium bromide staining of the gel.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Reverse Transcription System (AMV reverse transcriptase, oligo (dt) 15 and internal control, purchased from Promega) was used in transcription of DNA from the RNA samples. 1 μg total cellular RNA in 10 μl of nuclease-free dd-H_2O was added to RT mixture and incubated for 15 minutes at room temperature. It was then placed in the thermal cycler at 42 ^{0}C for 45 minutes, and then at 95 ^{0}C for 5 minutes.

Multiplex hot-start PCR was carried out with specific primers (c-*fos* and B-2 microglobulin) from Oligos, Inc. (15 pmol of each primer per reaction) in the Perkin

Elmer DNA Thermal Cycler 480 with the following protocol: 94 ^{0}C (5 min), 94 ^{0}C (1 min), 60 ^{0}C (1 min), and 72 ^{0}C (1 min) for 35 cycles; 72 ^{0}C (8 min). Hot-start PCR was done with AmpliWax PCR Gem 100 (Perkin Elmer). The total volume of each reaction was 75 μl. In addition to RT sample and primers, it also contained 5 units of AmpliTaq DNA polymerase, Stofell fragment (Perkin Elmer), 1X Stofell Buffer, 200 μM NTPs, 2.5 mM MgCl.

The amplification products were analyzed by 2% agarose gel electrophoresis containing 0.05 mg/ml ethidium bromide. The gel was photographed in UV-light. The negative was scanned and quantitated by Molecular Dynamic personal Densitometer (PDSI-486, Molecular Dynamics, Inc.) and analyzed by ImageQuant program.

The following primers were used:

For c-*fos* (22):

5'-GGC-TTC-AAC-GCA-GAC-TAC-GAG-GCG-T-3' (901-925)

5'-CCT-CCT-GCC-AAT-GCT-CTG-CGC-TCG-3' (1996-1973)

For beta-2 microglobulin (24):

5'-ATC-CAG-CGT-ACT-CCA-AAG-ATT-3' (943-949; 1340-1353)

5'-CAT-GTC-TCG-ATC-CCA-CTT-AAT-TAT-3' (1606-1618; 2235-2245)

Protein Extraction and Quantification

Protein extraction. The protocol was adapted from "Current Protocols in Molecular Biology". Flasks were put into an ice slurry immediately after exposure and

remained there for 10 minutes. HL-60 cells were collected by centrifugation at 1,500 rpm for 5 minutes. MCF-7 and 3Y1 cells were released from the bottom of the flasks by a cell scraper in the presence of 1X TBS with 0.2% of EDTA, and then spun down at 1,500 rpm for 5 minutes. After one rinse in 1X TBS, cells were lysed in 1X lysing buffer (1 ml per flask of HL-60 cells; 50 μl per flask of MCF-7 and 3Y1 cells) for 30 minutes on ice. Following vigorous vortexing, lysates were centrifuged at 14,000 rpm for 30 minutes at 4^0C and supernatants were saved for Western blotting analysis and immunoprecipitation.

Protein concentration determination. Total protein concentration of the samples was determined by Standard Protein DC Assay (Bio-Rad), a colorimetric assay for protein concentration following detergent solubilization. The reaction is similar to the well-documented Lowry assay. The reagent package includes: reagent A, an alkaline copper tartrate solution; reagent B, a dilute Folin reagent; and reagent S. Protein standards were prepared as a series of seven dilutions of bovine serum albumin with concentrations ranging from 1 to 7 μg/μl. Into a clean, dry cuvette, 10 μl of sample or protein standard was added along with 100 μl of reagent A' (1 part of reagent S + 50 parts of reagent A) and 800 μl of reagent B. After incubating at room temperature for 15 minutes, the absorbance was read at 710 nm. A standard curve would then be derived from the protein standards (Absorbance Vs Concentration), based on which protein concentration of the sample could be found.

Immunoprecipitation

Immunoprecipitation was performed on Raf-1. Total protein concentration for all samples was adjusted to 2 μg/μl before the start of the following procedure. 100 μl of whole cell lysate was incubated with 2 μg rabbit polyclonal anti-Raf-1 Ig (C-12, Santa Cruz Biotechnology), 400 μl H_2O, and 500 μl of 2X lysing buffer at 4^0C for 4 hours. 50 μl of 10% protein A-agarose (Calbiochem) was then added to the mixture. After vortex, the sample was incubated for 2 hours at 4^0C with agitation. The pellet was collected and washed with 1 X lysing buffer three times by centrifugation (5 minutes in an Eppendorf microcentrifuge). The proteins were released from the agarose beads by suspending the pellet in 30 μl of 2X SDS/sample buffer followed by 5 minutes' of boiling. After centrifugation for 5 minutes, the supernatant was ready for Western blotting analysis.

Gel Electrophoresis

Preparing of SDS-polyacrylamine gel. 7.5 ml separating gel solution (8%) was prepared with 2 ml of 30% acrylamide / N, N'- Methylene-bis-acrylamide (29:1) solution, 1.88 ml of 4 X Tris·Cl /SDS buffer (pH 8.8), and 3.62 ml of H_2O. The solution was then degassed for 15 minutes, and 25 μl of 10% ammonium persulfate and 10 μl of TEMED were added with gentle mixing. It was then immediately applied to the assembled glass plate sandwich (0.75 mm spacers were used) locked to a casting stand, and allowed to

polymerize for 30 minutes at room temperature. Water was added gently to shield the reaction away from the air.

5 ml stacking gel solution (4%) was prepared by mixing 0.65 ml of 30% acrylamide / Bis (29:1) with 1.25 ml of 4 X Tris·Cl /SDS buffer (pH 6.8) and 3.05 ml of H_2O. 25 µl of 10% ammonium persulfate and 10 µl of TEMED were added to the degassed (for 15 minutes) gel solution and mixed with a gentle swirl. After removal of the water seal, the stacking gel solution was applied on top of the solidified separating gel. A 0.75 mm Teflon comb was inserted right away into the layer of stacking gel as a mold for sample wells. After polymerizing for 30 minutes at room temperature, the gel was ready to use.

Gel electrophoresis. The gel sandwich was released from the gel caster and attached to the electrophoresis buffer chamber. The samples were prepared by mixing lysates (10-40 µg total protein) with 2 x (1:1 v/v) or 6 x (5:1 v/v) SDS/sample buffers. The mixtures were boiled for 5 minutes and then cool on ice. After removal of the Teflon comb, the samples (10-25 µl) and protein standards were loaded into the wells using a 100 µl syringe with a flat-tipped needle. The gel was run at 10 mA (DC) until the bromphenol blue tracking dye entered the separating gel. Then the DC current was increased to 15 mA. After the brompohenol blue tracking dye reached the bottom of the separating gel, the power was disconnected.

Western Blots

The sandwich was disassembled, and the gel was soaked in transfer buffer for a few minutes and then placed in an electroblot sandwich. The sandwich was put into a buffer chamber filled with transfer buffer. The proteins were transferred electrically from the gel to 0.45 μm nitrocellulose membrane (Bio-Rad) at 200 mA (DC) overnight in a 4 ^{0}C refrigerator.

After soaking in blocking buffer (5% nonfat milk powder in 1 x TBS or 1 x PBS) for an hour at room temperature, the membrane was incubated with primary antibody (diluted in blocking buffer) at 4 ^{0}C overnight with gentle agitation. The nitrocellulose membrane was then rinsed in wash buffer (1 X TBS + 0.1% Tween 20) three times before incubated with appropriate horseradish peroxidase-conjugated secondary antibody diluted in blocking buffer for an hour at room temperature with gentle agitation. After three rinses in washing buffer, the membrane was covered with ECL chemiluminescence (Amersham Life Sciences) Western blotting detection reagents for 5 minutes to allow the enzymatic reaction take place. By exposing the membrane to X-ray film, protein bands on the membrane could then be visualized. The density of the bands were measured with Molecular Dynamic personal Densitometer and analyzed by ImageQuant program.

Primary antibodies:

- Rabbit polyclonal anti-Raf-1 IgG (C-12, Santa Cruz Biotechnology), 1:2000 dilution
- Mouse monoclonal anti-Phosphotyrosine IgG2b$_k$ (clone 4G10, Upstate Biotechnology), 1:2000 dilution

- Mouse monoclonal anti-Phosphoserine IgG1 (clone PSR-45, Sigma), 1:500 dilution
- Mouse monoclonal anti-Phosphothreonine IgG2b (clone PTR-8, Sigma), 1:500 dilution
- Rat monoclonal anti-phospho-Raf-1 (Ser338) IgG1 (Upstate Biotechnology), 1:1000 dilution
- Mouse monoclonal anti-MAPK IgG1, activated (diphosphorylated ERK-1&2), clone MAPK-YT (Sigma), 1:1000 dilution

Chemicals

PKC inhibitors. PKC inhibitors Staurosporine, Gö 6976, and Rottlerin were purchased from Calbiochem and stock solutions were prepared by dissolving the chemicals in DMSO. Cells were incubated with Staurosporine, Gö 6976, and Rottlerin for 30 minutes prior to exposure at concentrations recommended by the manufacturer (0.2 μM, 1 μM, and 5 μM respectively). An equal amount of DMSO was added to the control flasks. The final concentration of DMSO in the medium was 0.1%.

Estrogen. Beta-estradiol 3-benzoate (EB) was purchased from Sigma and dissolved in DMSO. MCF-7 cells were treated with EB2 at different concentrations (0.1, 1, 10, and 100 nM). An equal amount of DMSO was added to control flasks. The final concentration of DMSO in the medium was 0.1%.

Statistical analysis

Data were analyzed statistically by Microsoft Excel Description Analysis Program. Student t-test was employed to determine if the differences between two samples (the population exposed to EMF vs. those not exposed) were significant. The test is based on the Null hypothesis which assumes that no difference exists between the two samples. If the p value (probability of an outcome) is greater than 0.05, then, by convention, the hypothesis is true. This particular test is very useful in deciding whether there is difference between two small samples.

CHAPTER III

RESULTS

Overview

The purpose of the present study was to explore the role of signal transduction pathways in EMF-cell interaction, focusing on the MAP kinase cascade, a crucial component of cell signaling system. It was initiated by our previous observations that differentiation of HL-60 cells from a nonphagocytic suspension culture to an attached fibroblast-like culture with high phagocytic activity elicited by TPA could be induced by exposure to a 60 Hz, 1 G EM field. Since transduction sequence leading to TPA induced cell differentiation has been well delineated (Nishizuka, 1986; Kharbanda *et al.*, 1994; Marquardt *et al.*, 1994; El-Shemerly *et al.*, 1997), this finding prompted us to investigate the transduction procession involved in EMF elicited cellular events using TPA induction pathway as a signaling model.

The impact of EMF on two members of the MAPK cascade, Raf-1 and MAP kinase, was tested to determine whether MAPK pathway could be induced by EMF. Experiments were done with MCF-7 and 3Y1 cells (parental cells and parental cells overexpressing c-Src) as well as with HL-60 cells. The results of our studies, as will be described in detail below, demonstrate that short-term (10 to 30 minutes) exposure to a 60 Hz, 1 G field leads to small but significant increases in the level of activation of both Raf-1 and MAPK in cells. These increases were seen with varying magnitude in all the cell lines tested and are comparable to those induced by low concentration of TPA (0.1 to 1 ng/ml). The existence of an additive effect between EMF and TPA on MAPK induction

was observed. Exposure to EMF in the presence of low concentrations of TPA elicited a more acute response in cells than treatment with either EMF or TPA alone. Furthermore, the role played by PKC in EMF induced signal transduction pathways was explored in inhibition studies where PKC inhibitors with different specificities were used. It seems that PKC-α rather than PKC-δ is required for the activation of MAPK cascade by EMF or TPA. Taken together, this study provides evidence that the MAPK pathway is affected by EM field exposure. As previous studies, this study indicates that EMF and TPA initiate a similar signal transduction pathway.

EMF and transcription of early express gene *c-fos*

Early expressed genes, such as *c-fos*, *c-jun*, and *c-myc*, encode transcription factors that are physiological targets of MAP kinase. They play a pivotal role in cell proliferation and differentiation. The expression of *c-fos* is enhanced by several stimuli including TPA (Fisch *et al.*, 1987), insulin, platelet derived growth factor, estrogen growth factor, and nerve growth factor (Doucet *et al.*, 1990). C-Fos combines with c-Jun to form the transcription factor AP1 that regulates the expression of genes involved in cell proliferation and differentiation, including *c-fos*.

The impact of EMF on the expression of *c-fos* has been investigated in several studies. Phillips *et al.* (1992) reported that the level of *c-fos* transcription increased 2.5 fold after CEM-CM3 cells were exposed to a 60 Hz, 0.1 mT field for 30 minutes. In our laboratory, a small, but significant change in the steady state mRNA level of *c-fos* was

detected by both northern blots and RT-PCR in HL-60 cells following EMF (60 Hz, 60 mG) exposure for 20 minutes.

The present experiment was set to confirm these previous findings. TPA (10 ng/ml) was used as a positive control in the study. The expression of *c-fos* was determined by RT-PCR.The expression of β2 microglobulin served as an internal control. The data (Figure 6) revealed a transient increase in the level of *c-fos* transcription in HL-60 cells after short-term exposure to EMF, starting at 15 minutes and culminating at 20 minutes. Compared to TPA, EMF seems to be a weaker stimulus of *c-fos* gene. These observations are inconsistent with those made before.

The fact that *c-fos* could be induced by EMF field exposure was the first strong proof that signaling processes could be involved. It was a corner stone in planning other experiment to determine the role of signaling in cellular response to EMF.

EMF and Raf-1 activation

Raf-1 can be phosphorylated at multiple residues and hyperphosphorylation is always related to Raf-1 activation. In the following experiments, two approaches were employed to determine indirectly the activation state of Raf-1 in cells exposed to EM field: measurement of the level of tyrosine phosphorylation in general and phosphorylation of a specific residue, Ser338.

Tyrosine phosphorylation of Raf-1 in HL-60 cells exposed to EMF. There are studies that correlate tyrosine phosphorylation with Raf-1 activity (Turner *et al.*, 1992).

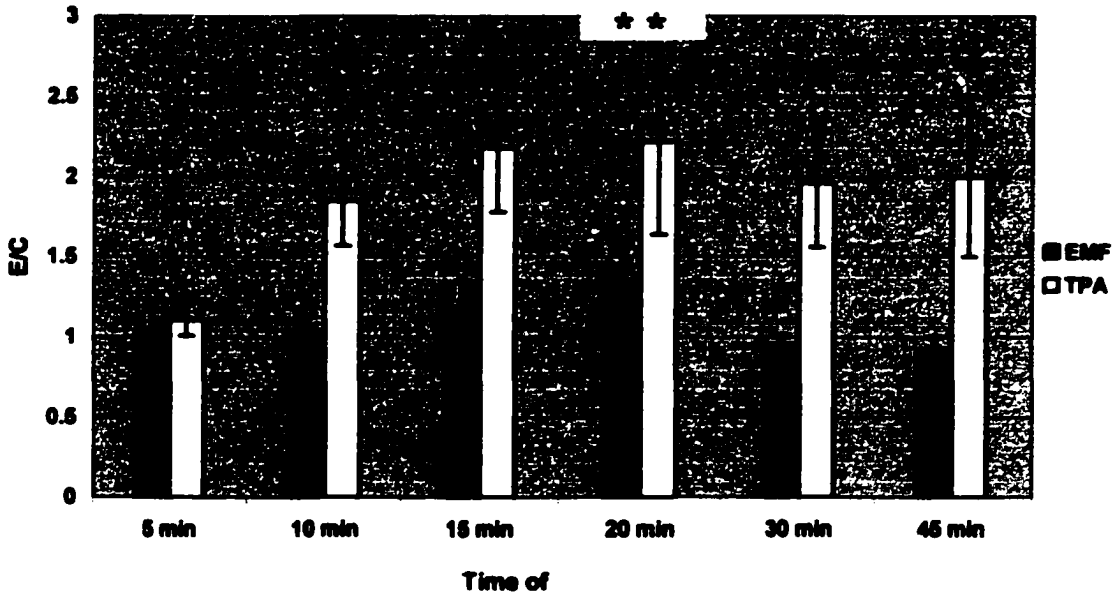


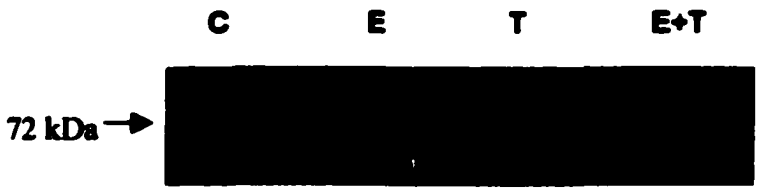
Figure 6. ***C-fos* gene expression in HL-60 cells exposed to EMF.** HL-60 cells were exposed to a 60 Hz, 60 mG EM field or treated with 10 ng/ml of TPA. The expression of *c-fos* gene was detected by RT-PCR. The values of the bars (E/C) are the ratios of levels of c-fos gene expression in experimental samples (E) over those of the controls. E/C is adjusted to the expression of β-microglobulin gene of the sample (* indicates significant difference from control). Three experiments were done with different treatments for all time points tested.

In this group of experiments, tyrosine phosphorylation of Raf-1 was detected with the following procedure: Raf-1 in the lysate of treated cells was immunoprecipitated by anti-Raf-1, and then the resulting immunocomplex was subjected to western blots analysis with an anti-phosphotyrosine antibody. The amount of Raf-1 protein in the samples was monitored by western blots with anti-Raf-1.

The results show that the amount of Raf-1 in cells is not affected by either EMF exposure or TPA treatment (Figure 7). Yet a small (about 30%), but significant increase in the level of tyrosine phosphorylation was observed after 10 minutes of exposure to EMF or TPA (Figure 8). Increases were also detected following 2.5 and 20 minutes of exposure, but were not statistically significant. Similar trend was seen when level of Raf-1 tyrosine phosphorylation was normalized by the amount of Raf-1 protein (Figure 9). These data suggest that Raf-1 might be activated by EMF as well as by TPA. Moreover, it seems that there exists a correlation between EMF and TPA induced Raf-1 tyrosine phosphorylation relative to time of exposure, although a synergy or additive effect between EMF and TPA is unlikely.

Another notable fact demonstrated in these experiments is that the enhancement in the level of tyrosine phosphorylation after TPA treatment was relatively small considering the sometimes visible retardation of Raf-1 in SDS-PAGE (Figure 10), which signifies the hyperphosphorylation of Raf-1 protein, and the increase in the level of MAPK activation in response to the same concentration of TPA (Figure 8). It indicates that tyrosine phosphorylation of Raf-1 may play only a minor role in TPA induced MAPK pathway and that phosphorylation of certain serine and threonine residues of Raf-1 might be involved. This could also apply to EMF induction to a lesser extent.

A



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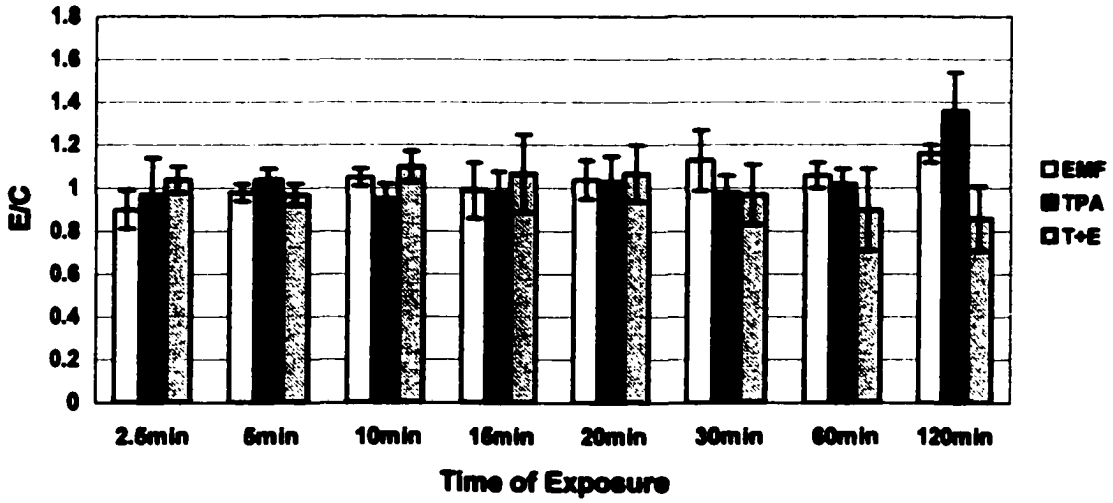

Figure 7. **The amount of Raf-1 protein in HL-60 cells exposed to EMF. A:** HL-60 cells were exposed to a 60 Hz EMF (E) at 1 G, treated with 1 ng/ml of TPA (T) or subjected to both EMF and TPA (T+E) treatment for 10 minutes (C: control). Raf-1 in cell lysates was detected by Western blots with polyclonal rabbit anti-Raf-1 (C-12). **B:** The graph summarizes a series of timed experiments as described in A. The density of the bands (C: control, E: experiment) was determined by densitometry. The data were normalized by the amount of total protein. The values of the bars (E/C) represent the ratios of the amount of Raf-1 protein in experimental samples over that of the controls. The numbers of experiment for different time points of different treatments are: E (2.5 min – 7, 5 min – 6, 10 min – 12, 15 min – 9, 20 min – 8, 30 min – 7, 60 min – 3, 120 min – 3); T (2.5 min – 7, 5 min – 6, 10 min – 10, 15 min – 7, 20 min – 7, 30 min – 7, 60 min – 3, 120 min – 3); T+E (2.5 min – 6, 5 min – 6, 10 min – 8, 15 min – 6, 20 min – 7, 30 min – 6, 60 min – 3, 120 min – 3).

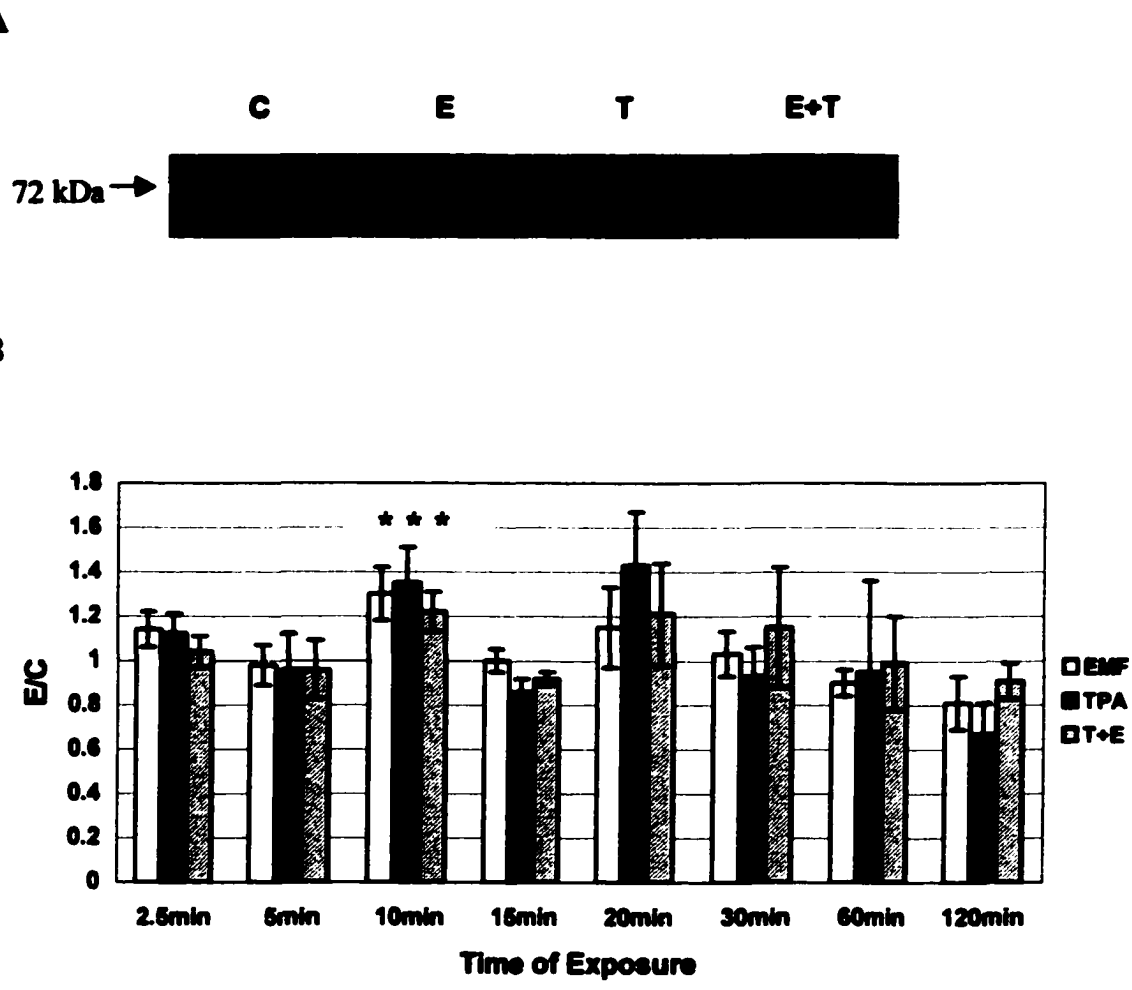

Figure 8. **Effects of EMF exposure on Raf-1 tyrosine phosphorylation in HL-60 cells.** **A:** HL-60 cells were exposed to a 60 Hz EMF (E) at 1 G, treated with 1 ng/ml of TPA (T) or exposed to EMF in the presence of TPA (T+E) for 10 minutes (C: Control). Raf-1 in cell lysates was immunoprecipitated with anti-Raf-1 and then tyrosine phosphorylation of Raf-1 was detected by Western blots performed on the immunoprecipitation complex against monoclonal anti-phosphotyrosine. **B:** The graph summarizes a series of timed experiments as described in A. The density of the bands (C: control, E: experiment) was determined by densitometry. The data were normalized by the amount of total protein. The values of the bars (E/C) represent the ratios of the tyrosine phosphorylation levels of Raf-1 in experimental samples over those of the controls (* indicates significant difference from control). The numbers of experiment for different time points of different treatments are: E (2.5 min – 7, 5 min – 7, 10 min – 13, 15 min – 10, 20 min – 8, 30 min – 7, 60 min – 3, 120 min – 3); T (2.5 min – 7, 5 min – 7, 10 min – 11, 15 min – 8, 20 min – 7, 30 min – 7, 60 min – 3, 120 min – 3); T+E (2.5 min – 6, 5 min – 5, 10 min – 9, 15 min – 7, 20 min – 7, 30 min – 5, 60 min – 3, 120 min – 3).

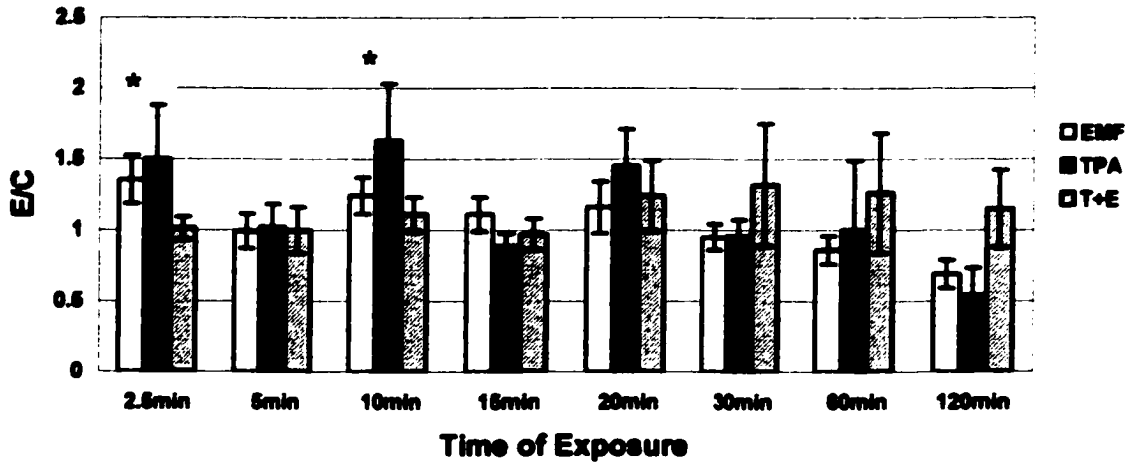

Figure 9. Level of Raf-1 tyrosine phosphorylation as normalized by the amount of Raf-1 protein in HL-60 cells exposed to EMF. The E/C values presented in Figure 8B were adjusted to the amount of Raf-1 in the samples (* indicates significant difference from control). The numbers of experiment for different time points of different treatments are: E (2.5 min – 7, 5 min – 6, 10 min – 12, 15 min – 9, 20 min – 8, 30 min – 7, 60 min – 3, 120 min – 3); T (2.5 min – 7, 5 min – 6, 10 min – 10, 15 min – 7, 20 min – 7, 30 min – 7, 60 min – 3, 120 min – 3); T+E (2.5 min – 6, 5 min – 5, 10 min – 8, 15 min – 6, 20 min – 7, 30 min – 5, 60 min – 3, 120 min – 3).

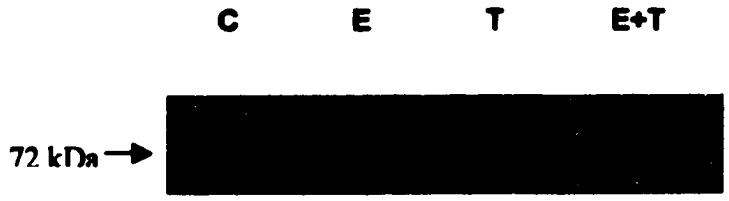

Figure 10. **TPA causes migration retardation of Raf in SDS-PAGE.** HL-60 cells were exposed to a 60 Hz EMF (E) at 1 G, treated with 1 ng/ml of TPA (T) or exposed to EMF in the presence of TPA (T+E) for 10 minutes (C: Control). Raf-1 in cell lysates was detected by Western blots with polyclonal rabbit anti-Raf-1 (C-12).

Attempts were made to determine the general state of serine and threonine phosphorylation of Raf-1 in HL-60 subjected to TPA or EMF exposure. Anti-phosphoserine and anti-phosphothreonine antibodies were used following the same protocols as in previous experiments. No serine or threonine phosphorylation of Raf-1 was detected after exposure to either TPA or EMF, probably due to the inaccessibility of the phosphorylated serine and threonine residues to the antibodies, *i.e.* serine and threonine residues are shielded away from the antibodies (the explanation appears in the Sigma catalogue).

Phosphorylation at Ser338 of Raf-1 in cells exposed to EMF. Phosphorylation on specific tyrosine, serine, or threonine residues has significant bearing on regulating Raf-1 activity, and could be elicited by extra-cellular stimuli such as insulin, PDGF, EGF, interleukin and TPA. Increased level of Raf-1 phosphorylation on one such residue, Ser 338, has been reported in COS cells treated with EGF, TPA or PDGF, and there is evidence that this phosphorylation event is required for growth factor stimulated Raf-1 activation (Mason *et al.*, 1999; Zang *et al.*, 2001).

Since it has been shown that the effect of EMF on tyrosine phosphorylation of Raf-1 in HL-60 cell is rather small, tests with utmost sensitivity are needed to detect any changes resulting from EMF exposure. In this group of experiments, phosphorylation on Ser338 of Raf-1 in HL-60 and MCF-7 cells exposed to EMF was investigated with an experimental approach different from the previous ones. An antibody specific for only the Ser338 phosphorylated Raf-1 was used in direct immunoblotting detection without the added step of immunoprecipitation that might introduce more deviation and contribute to the fluctuation of the data (Figure 8 & Figure 9).

Increased level of phosphorylation on Ser338 of Raf-1 was detected following EMF exposure in both cell lines (Table 1 and Figure 11). It seems that MCF-7 cells are more sensitive to EM field than HL-60 cells. Over 100% increase in Raf-1 phosphorylation on Ser338 was seen in MCF-7 cells exposed to the field as compared to that of 30% increase in HL-60 cells. These data provide further evidence that Raf-1 could be induced by EMF.

EMF and MAP kinase activation

To further the investigation of the involvement of MAP kinase cascade in cellular responses to EMF exposure, this experimental series focused on the impact of EM field on another member of the MAPK cascade that functions down stream of Raf-1, MAP kinase or ERK, in three different cell lines: HL-60, MCF-7, and 3Y1 (parental cells and parental cells transfected by either c-Src or v-Src) cells.

Rat fibroblast 3Y1 cells used in this experiment respond positively to TPA stimulation. The parental cells and cells over-expressing the nontransforming c-Src proto-oncogene or the oncogenic v-Src display distinct morphologies among themselves in cultured conditions, with v-Src transfected cells exhibiting an anchorage-independent growth which is an indication of transformed phenotype. It has been shown that prolonged exposure to TPA could induce in cells transfected by c-Src a change in morphology towards that displayed by v-Src-transformed cells that results in an anchorage-independent growth of these cells, while no morphological change was seen in

Cell Lines	Duration of Exposure	Type of Exposure	Number of Exposures	E/C: Mean ± SE	P (Differ from 1)
HL-60	15 min	EMF	5	1.31 ±0.08	0.009
		TPA	2	1.14 ±0.04	0.08
		EMF+TPA	2	1.47 ±0.3	0.18
MCF-7	10 min	EMF	3	1.67 ±0.22	0.5
		TPA	3	1.99 ±0.52	0.1
		EMF+TPA	3	2.18 ±0.86	0.15
	15 min	EMF	5	2.02 ±0.35	0.02
		TPA	3	2.42 ±0.35	0.03
		EMF+TPA	3	2.56 ±0.45	0.04
	20 min	EMF	3	1.89 ±0.38	0.07
		TPA	3	1.92 ±0.2	0.02
		EMF+TPA	3	2.05 ±0.28	0.03

Table 1. **Phosphorylation of Raf-1 on Ser338 in HL-60 and MCF-7 cells exposed to EMF.** HL-60 and MCF-7 cells were exposed to a 60 Hz EMF at 1 G, treated with 0.1 ng/ml of TPA or exposed to EMF in the presence of TPA for durations indicated. Raf-1 phosphorylation on Ser338 in cell lysates was detected by immunoblotting with rat monoclonal anti-phospho-Raf-1 (Ser338) that only recognizes the Ser338 phosphorylated Raf-1. The density of the bands (C: control, E: experiment) was determined by densitometry. The data were normalized by the amount of total protein. E/C represents the ratio of level of Raf-1 phosphorylation on Ser338 in experimental sample over that of the control.

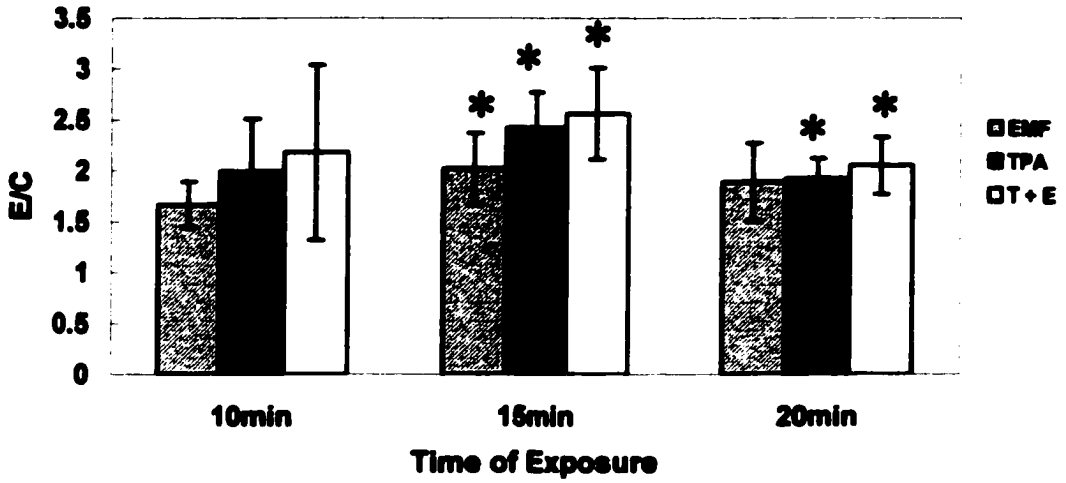

Figure 11. **Effects of EMF on phosphorylation of Raf-1 at Ser338 in MCF-7 cells.** MCF-7 cells were exposed to a 60 Hz EMF (E) at 1 G, treated with 0.1 ng/ml of TPA (T) or exposed to EMF in the presence of TPA (T+E) for 10, 15, and 20 minutes. Raf-1 phosphorylation on Ser338 in cell lysates was detected by immunoblotting with rat monoclonal anti-phospho-Raf-1 (Ser338) that only recognizes the Ser338 phosphorylated Raf-1. The density of the bands (C: control, E: experiment) was determined by densitometry. The data were normalized by the amount of total protein. The values of the bars (E/C) represent the ratios of the levels of Raf-1 phosphorylation on Ser338 in experimental sample over those of the controls (* indicates significant difference from control). The numbers of experiment for different time points of different treatments are: E (10 min – 3, 15 min – 5, 20 min – 3); T (10 min – 3, 15 min – 3, 20 min – 3); T+E (10 min – 3, 15 min – 3, 20 min – 3).

parental 3Y1 cells subjected to the same treatment (Lu *et al.*, 1997). On the strength of the above-mentioned qualities, this cell line could serve as a model in the study of the role of EMF exposure in tumor promotion, with v-Src transformed cells as internal positive controls and c-Src transfected cells representing pre-cancerous cells. That is why the effects of EMF on signal transduct of 3Y1 cells, in this case the impact of field exposure on MAPK activation, were of particular interest to us.

Our research also extent to MCF-7 cells because recent studies in several laboratories demonstrate that exposure to power frequency electromagnetic fields offsets the antiproliferative effects of melatonin and tamoxifen in these cells (Harland *et al.*, 1999; Blackman *et al.*, 2001), and this impact of EMF may well be mediated via MAPK pathway.

MAP kinase is activated in mitogen-stimulated cells only when both of the two onserved residues, a threonine and a tyrosine, (one residue from each other, e.g., Thr183 and Tyr185 in ERK2), within the regulatory site are phosphorylated by MEK (Ahn *et al.*, 1992; Seger *et al.*, 1992). A highly specific antibody that recognizes only the active dually-phosphorylated form of MAPK (ERK-1 and ERK-2, 44 kD and 42 kD, respectively) was used to determine the activation state of MAPK in cells exposed to EMF by western blots.

Activation of MAP kinase by TPA. Previous studies (Kharbanda *et al.*, 1994) reported that MAPK was activated in HL-60 cells treated with TPA. The present research confirmed this observation. In addition, it showed that MAPK was induced by TPA in a dosage dependent way in HL-60, MCF-7, 3Y1, and c-Src transfected 3Y1 cells. V-Src transfected 3Y1 has a level of MAPK activation that is extremely high with or without

TPA (Figure 12), indicating that this pathway is turned on permanently in these cells due to the overexpression of v-Src gene. This is consistent with reports that MAPK was activated constitutively in cells transformed by v-Src (Gupta *et al.*, 1992). The dosage response curves of MAPK activation versus TPA treatment (15 minutes) in 3Y1, c-Src, transfected 3Y1, MCF-7, and HL-60 cells are presented in Figures 13, 14, 15, 16 respectively. Cells were treated with TPA for varying durations (zero to 30 minutes) and a robust response was elicited within 15 minutes of TPA treatment in all four cell lines.

MAP activation in cells exposed to EMF. An increased level of MAPK activation was also observed in HL-60, MCF-7, 3Y1, and c-Src expressing 3Y1 (c-Src) cells exposed to 60 Hz, 1 G EMF for 10-30 minutes with some variation in time of response between different cell lines (Figure 17). MCF-7 cells exhibited the strongest response to EMF. Significant increases in MAPK activation were seen after 10, 15, 20, and 30 minutes of exposure and peaked at 15 minutes at a level over two-folds of that seen in control cells. The increase was significant at 15 minutes in HL-60 cells; while in 3Y1 and c-Src cells, significant increases were found after 10, 15, and 20 minutes of EMF exposure. The activation culminated at 10 minutes for 3Y1 cells and 15 and 20 minutes for c-Src. It is worth mentioning that the enhancement in MAPK activation detected in HL-60 and MCF-7 cells exposed to EMF seems to correlate with the increase in the level of phosphorylation on Ser338 of Raf-1 in these two cell lines resulting from field exposure in terms of both magnitude and time dependency (Figure 11 and Table 1). This suggests that the activations of these kinases are two closely related events and take place immediately.

Figure 12. **Activation state of MAP kinase in 3Y1 cells transfected with v-Src.** V-Src transfected 3Y1 cells were exposed to a 60 Hz EMF (E) at 1 G, treated with 1 ng/ml of TPA (T) or exposed to EMF in the presence of TPA (T+E) for 10 minutes (C: Control). Cell lysates were subjected to Western blotting analysis against monoclonal anti-MAP kinase, activated (di-phosphorylated MAPK/ERK-1 & 2, 42 & 44 kDa). The density of the bands indicates the activity of the kinase.

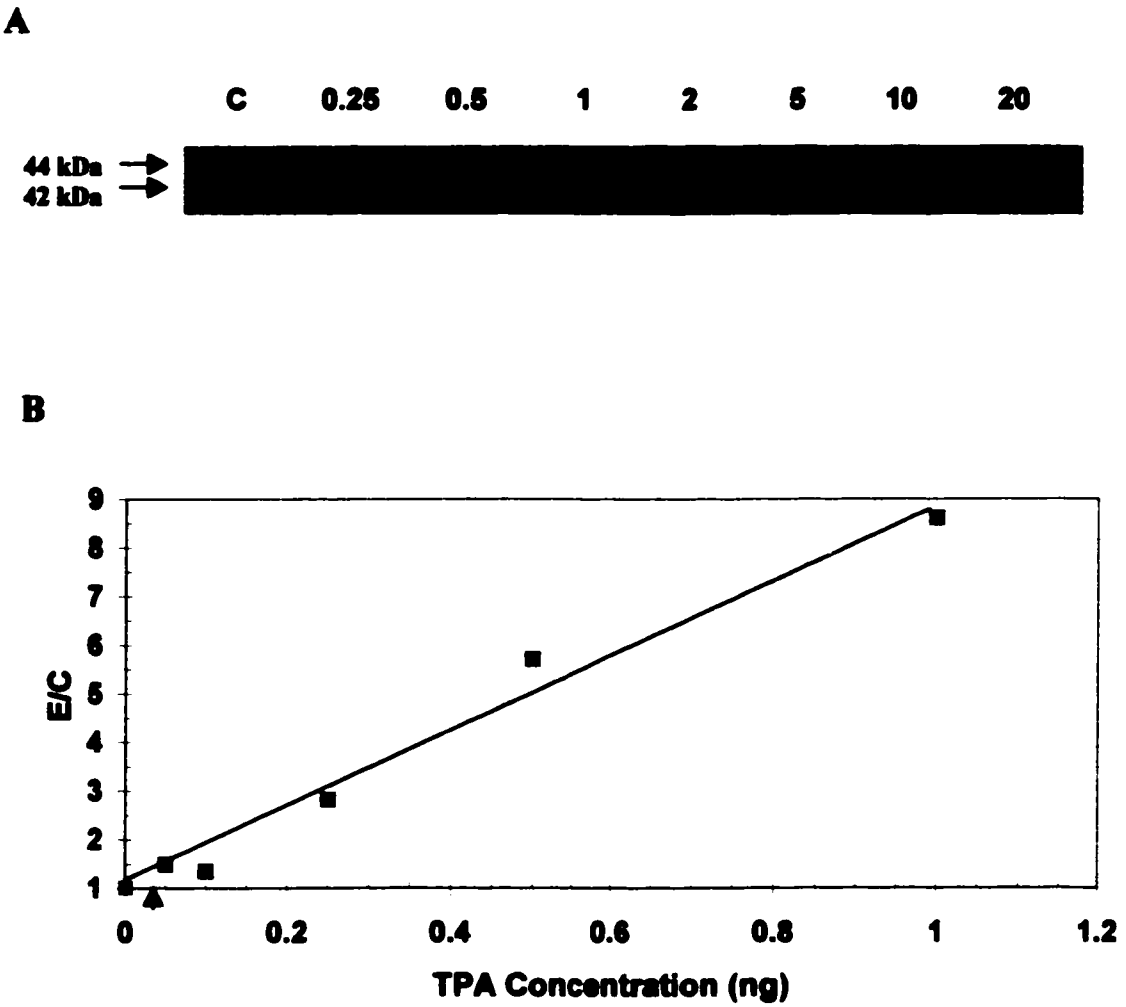

Figure 13. **TPA-induced activation of MAPK in 3Y1 cells. A.** 3Y1 cells were treated with 0.25, 0.5, 1, 2, 5, 10, 20 ng/ml of TPA for 15 minutes (C: control, no treatment). Activated MAPK in the cell lysates was detected as described in Figure 12. The density of the bands indicates level of MAPK activation. **B.** The graph summarizes a series of experiments as described in A. The density of the bands (42 & 44 kDa; C: control; E: experiment) was determined by densitometry. The data were normalized by the amount of total protein. The ratio, E/C, allows a comparison of level of MAPK activation in 3Y1 cells treated with different concentration of TPA. The arrow points to the TPA concentration that elicited the same level of activation of MAPK in 3Y1 cells as that induced by exposure to EMF (60 Hz, 1 G).

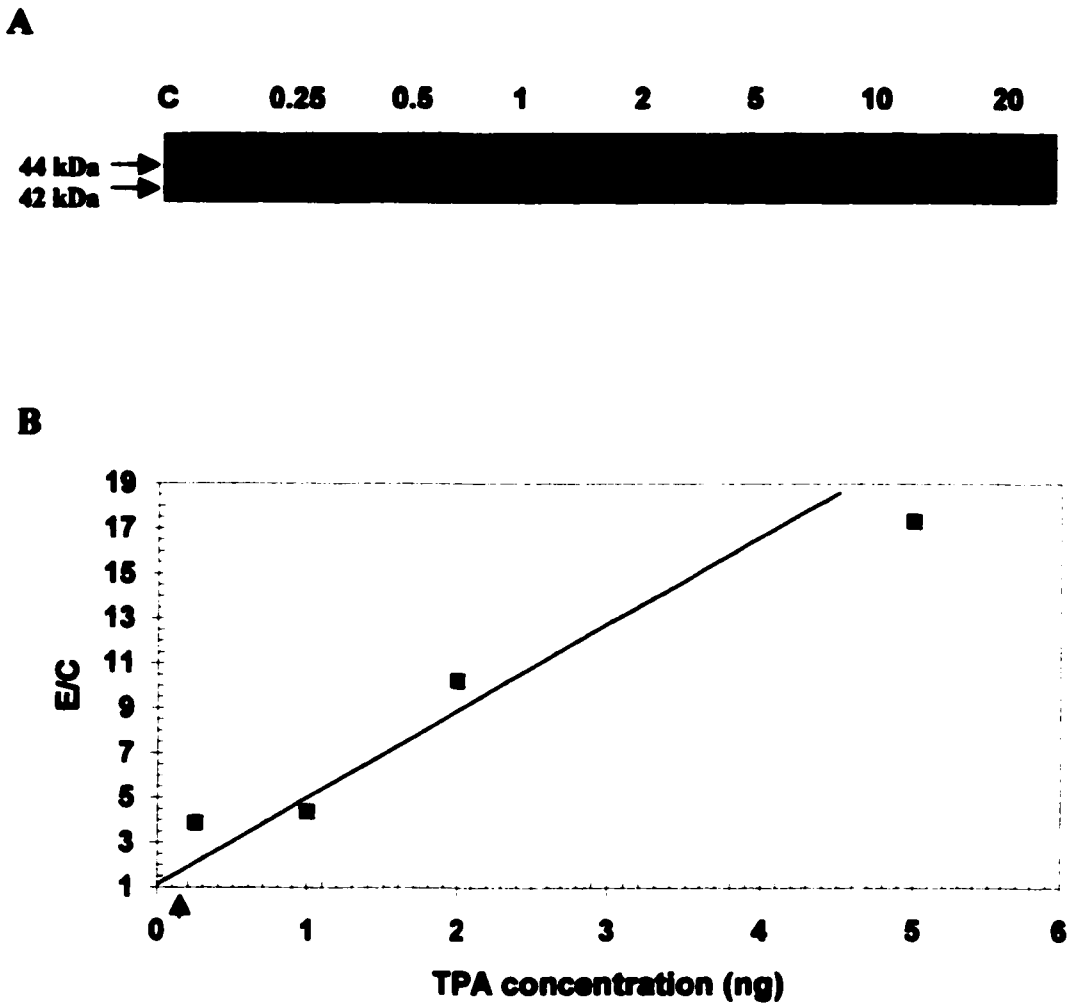

Figure 14. **TPA-induced activation of MAPK in 3Y1 cells transfected with c-Src. A.** C-Src transfected 3Y1 cells were treated with 0.25, 0.5, 1, 2, 5, 10, 20 ng/ml of TPA for 15 minutes (C: control, no treatment). Activated MAPK in the cell lysates was detected as described in Figure 12. The density of the bands indicates level of MAPK activation. **B.** The graph summarizes a series of experiments as described in A. The density of the bands (42 & 44 kDa; C: control; E: experiment) was determined by densitometry. The data were normalized by the amount of total protein. The ratio, E/C, allows a comparison of level of MAPK activation in c-Src transfected 3Y1 cells treated with different concentration of TPA. The arrow points to the TPA concentration that elicited the same level of activation of MAPK in c-Src transfected 3Y1 cells as that induced by exposure to EMF (60 Hz, 1 G).

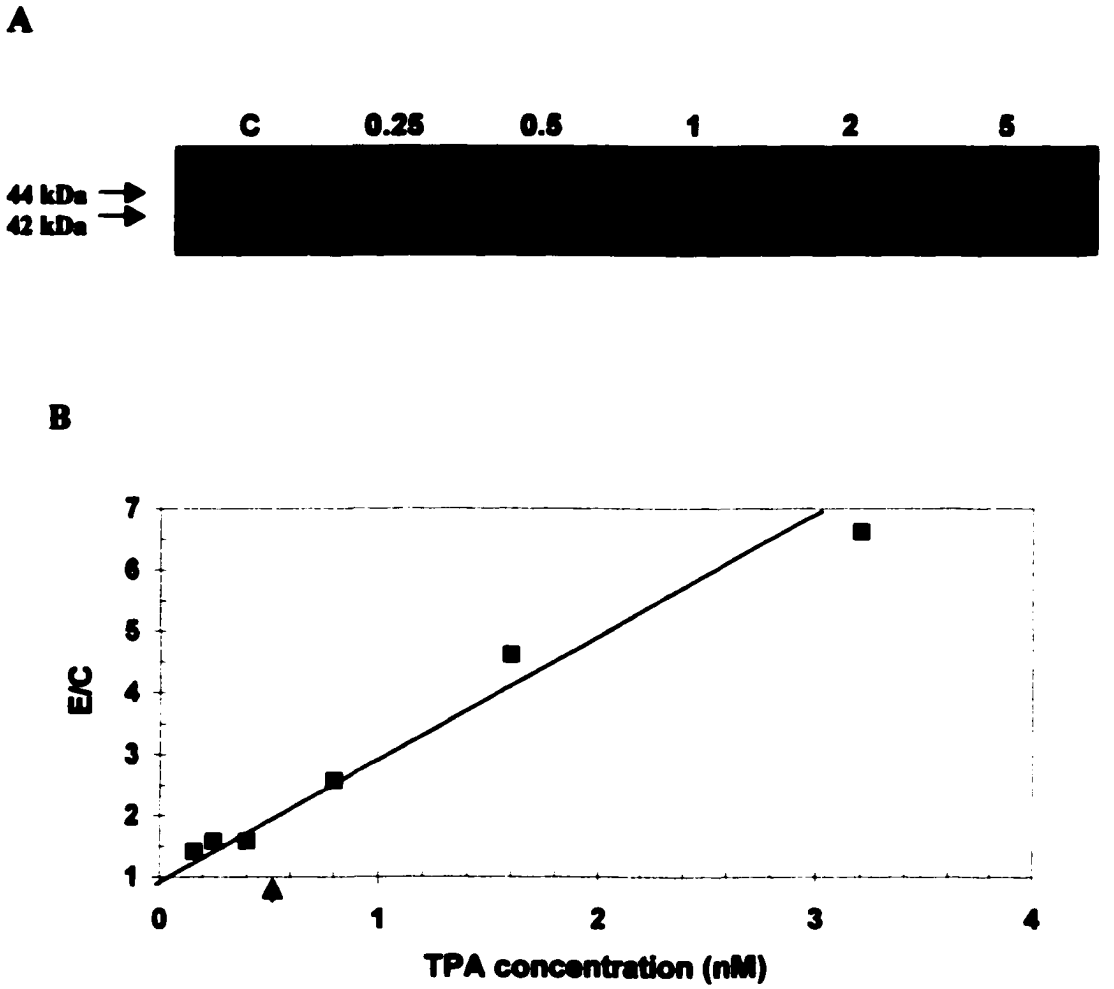

Figure 15. **TPA-induced activation of MAPK in MCF-7 cells. A.** MCF-7 cells were treated with 0.25, 0.5, 1, 2, 5 ng/ml of TPA for 15 minutes (C: control, no treatment). Activated MAPK in the cell lysates was detected as described in Figure 12. The density of the bands indicates level of MAPK activation. **B.** The graph summarizes a series of experiments as described in A. The density of the bands (42 & 44 kDa; C: control; E: experiment) was determined by densitometry. The data were normalized by the amount of total protein. The ratio, E/C, allows a comparison of level of MAPK activation in MCF-7 cells treated with different concentration of TPA. The arrow points to the TPA concentration that elicited the same level of activation of MAPK in MCF-7 cells as that induced by exposure to EMF (60 Hz, 1 G).

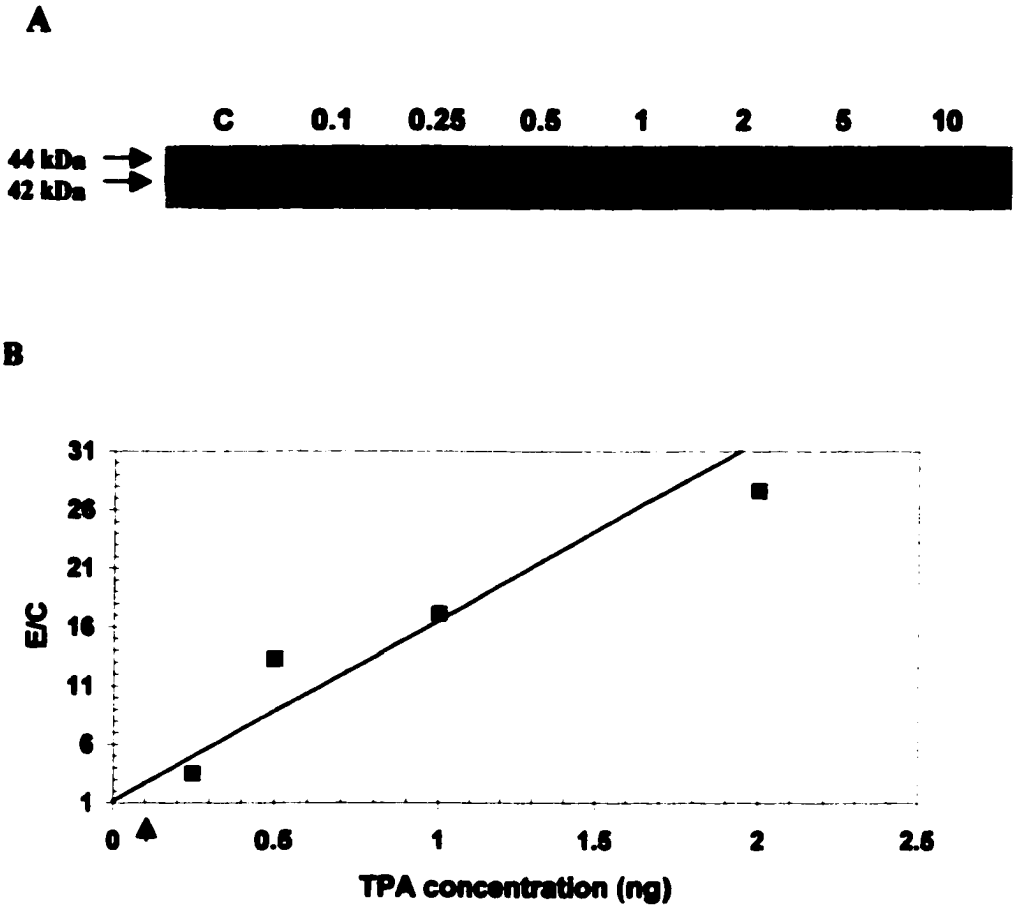

Figure 16. **TPA-induced activation of MAPK in HL-60 cells. A.** HL-60 cells were treated with 0.1, 0.25, 0.5, 1, 2, 5, 10 ng/ml of TPA for 15 minutes (C: control, no treatment). Activated MAPK in the cell lysates was detected as described in Figure 12. The density of the bands indicates level of MAPK activation. **B.** The graph summarizes a series of experiments as described in A. The density of the bands (42 & 44 kDa; C: control; E: experiment) was determined by densitometry. The data were normalized by the amount of total protein. The ratio, E/C, allows a comparison of level of MAPK activation in HL-60 cells treated with different concentration of TPA. The arrow points to the TPA concentration that elicited the same level of activation of MAPK in HL-60 cells as that induced by exposure to EMF (60 Hz, 1 G).

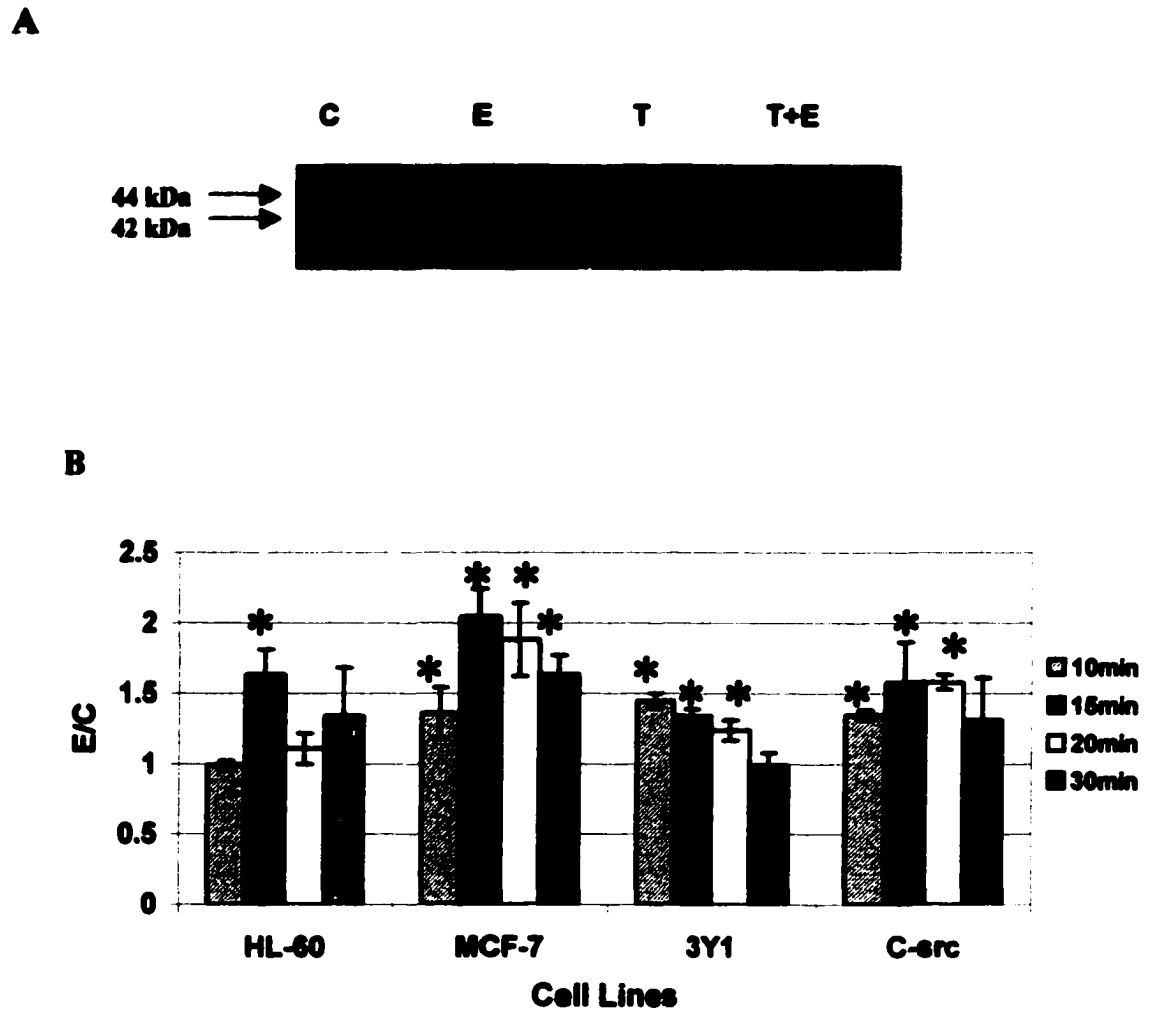

Figure 17. **Effects of EMF exposure on MAPK activation in different cell lines. A:** MCF-7 cells were exposed to a 60 Hz EMF (E) at 1 G, treated with 1 ng/ml of TPA (T) or exposed to EMF in the presence of TPA (T+E) for 20 minutes (C: Control). Activated MAPK in the cell lysates was detected as described in Figure 12. The density of the bands (42 & 44 kDa) that indicates level of MAPK activation was determined by densitometry. **B:** The graph presents the results of exposures of HL-60, MCF-7, 3Y1, c-Src transfected 3Y1 cells to 60 Hz, 1 G EMF for 10, 15, 20, and 30 minutes. The data were normalized by the amount of total protein. The values of the bars (E/C) represent the ratios of the MAPK activation levels in experimental samples (E) over those of the controls (C) as determined by Western blots (* indicates significant difference from control). The numbers of experiment for different time points of different cell lines are: HL-60 cells (10 min – 3, 15 min – 13, 20 min – 4, 30 min – 4); MCF-7 cells (10 min – 6, 15 min – 15, 20 min – 6, 30 min – 3); 3Y1 cells (10 min – 3, 15 min – 4, 20 min – 3, 30 min – 3); c-Src transfected 3Y1 cells (10 min – 3, 15 min – 6, 20 min – 3, 30 min – 3).

In all of the cell lines tested in the experiments, a rather small increase in MAPK activation was induced by EMF exposure considering the maximum increase that could be elicited by TPA (50-100 folds when cells were treated with TPA of concentration in excess of 50 ng/ml). The level of MAPK activation in cells exposed to EMF was comparable only to that observed in cells treated with low concentration of TPA (0.05-0.6 ng/ml). While MCF-7 was the cell line most sensitive to EMF, its response to TPA was relatively weaker when compared with that of other cell lines. In MCF-7 cells, EMF exposure was equivalent to treatment with 0.5-0.6 ng/ml of TPA, while in HL-60, 3Y1 and c-Src cells, EMF exposure has the same impact as that produced by 0.05-0.1 ng/ml of TPA (Figure 13, 14, 15, 16).

Comparison of the effects of EMF and TPA on MAPK activation. The similar response seen in cells exposed to EMF or TPA again raises the question of whether these two extracellular stimuli share the same signal transduction pathway to propagate the signal initiated at the cell surface to the nucleus. A series of timed experiments (zero to 30 minutes) comparing the effects of EMF exposure with TPA treatment (0.25 ng/ml) on MAPK in HL-60, MCF-7, 3Y1, and c-Src transfected 3Y1 cells was conducted to determine whether they follow similar time frames in MAPK induction. The data suggest that MAPK were affected by either treatment, but it seems that cells sustain their responsiveness for a longer period to TPA than to EMF in terms of MAPK activation (Figure, 18, 19, 20, 21). Minor differences exist between different cell lines in their reaction to EMF exposure and TPA treatment, but overall, MAPK activation induced by EMF or TPA demonstrates a similar trend among all of them (Figure 22). These

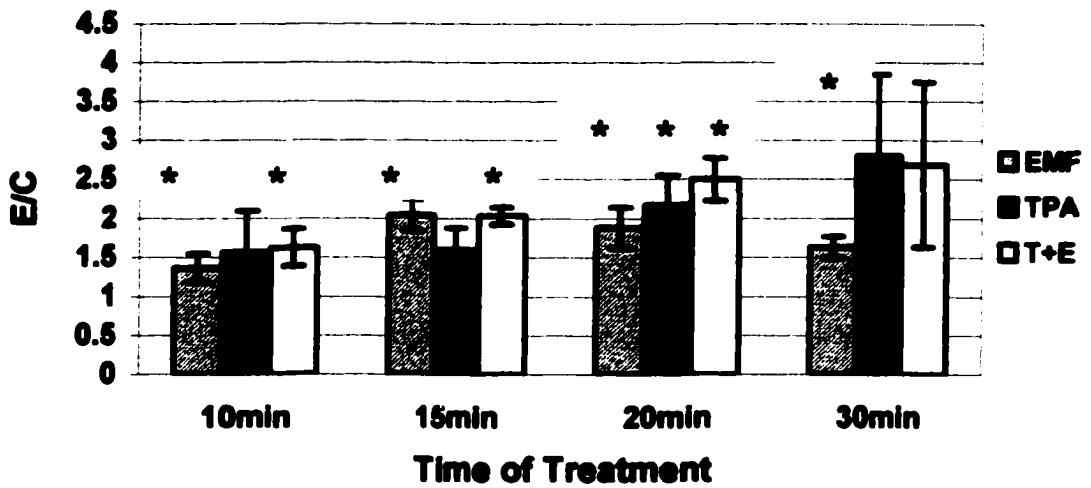

Figure 18. **Effects of EMF exposure, TPA treatment, and EMF exposure in the presence of TPA on MAPK activation in MCF-7 cells over time.** MCF-7 cells were exposed to EMF (60 Hz, 1 G), TPA (0.25 ng /ml), or both EMF and TPA (E+T) for 10, 15, 20, and 30 minutes. The levels of the activated form of MAPK in the cell lysates were determined by Western blots as described in Figure 12. The data were normalized by the amount of total protein. The values of the bars (E/C) are the ratios of levels of MAPK activation in experimental samples (E) over those the controls (C). * Indicates significant difference from control. The numbers of experiment for different time points of different treatments are: E (10 min – 6, 15 min – 15, 20 min – 6, 30 min – 3); T (10 min – 3, 15 min – 3, 20 min – 3, 30 min – 3); T+E (10 min – 4, 15 min – 3, 20 min – 3, 30 min – 3).

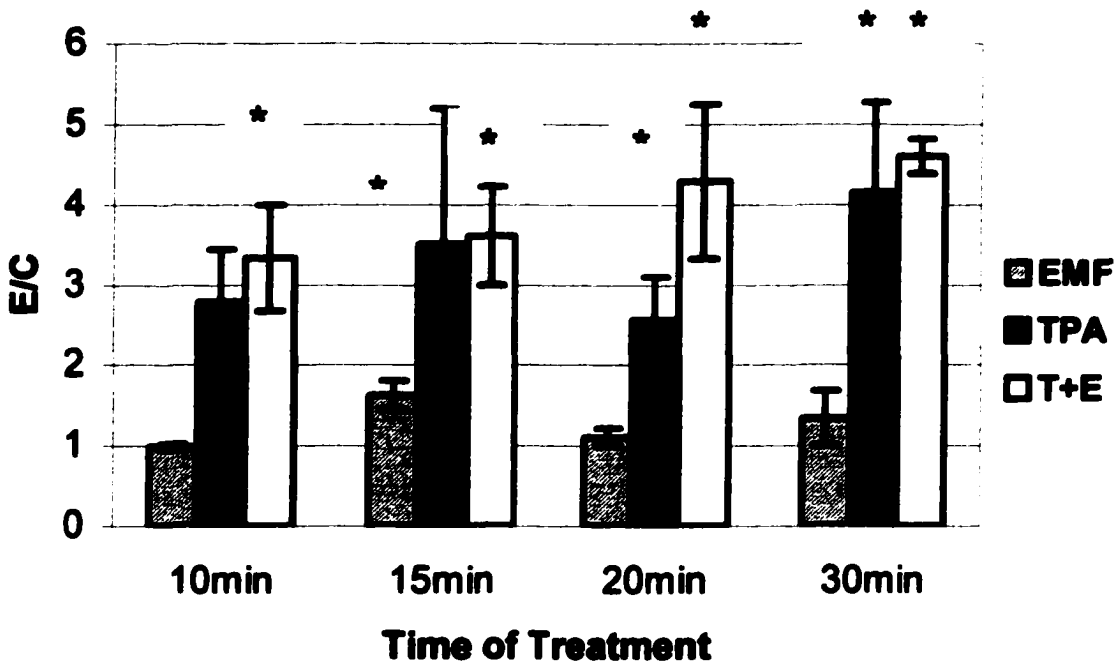

Figure 19. **Effects of EMF exposure, TPA treatment, and EMF exposure in the presence of TPA on MAPK activation in HL-60 cells over time.** HL-60 cells were exposed to EMF (60 Hz, 1 G), TPA (0.25 ng /ml), or both EMF and TPA (E+T) for 10, 15, 20, and 30 minutes. The levels of the activated form of MAPK in the cell lysates were determined by Western blots as described in Figure 12. The data were normalized by the amount of total protein. The values of the bars (E/C) are the ratios of levels of MAPK activation in experimental samples (E) over those the controls (C). * Indicates significant difference from control. The numbers of experiment for different time points of different treatments are: E (10 min – 3, 15 min – 13, 20 min – 4, 30 min – 4); T (10 min – 3, 15 min – 4, 20 min – 3, 30 min – 3); T+E (10 min – 3, 15 min – 3, 20 min – 3, 30 min – 3).

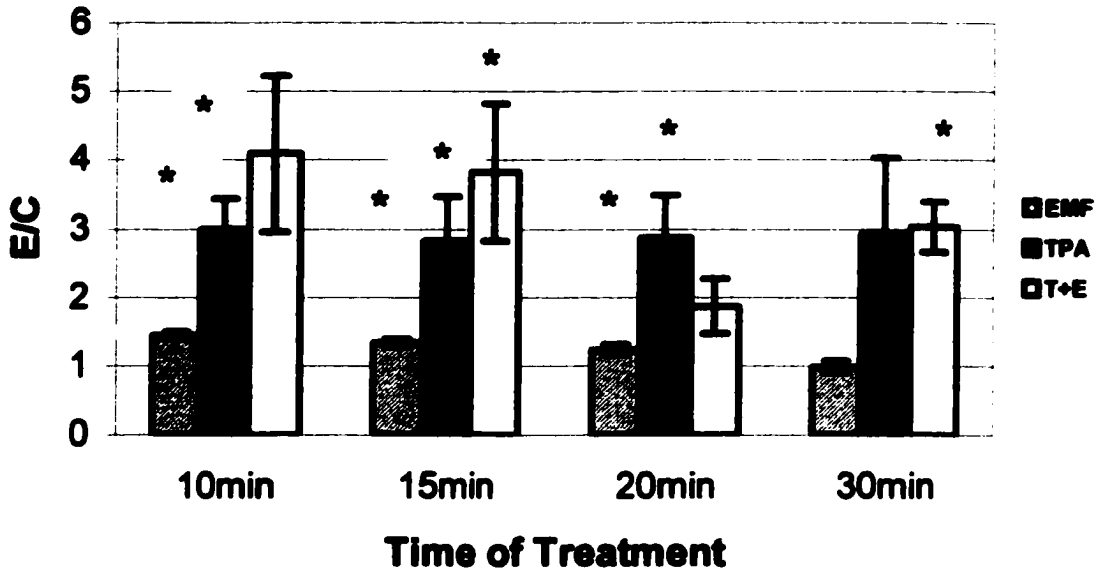

Figure 20. **Effects of EMF exposure, TPA treatment, and EMF exposure in the presence of TPA on MAPK activation in 3Y1 cells over time.** 3Y1 cells were exposed to EMF (60 Hz, 1 G), TPA (0.25 ng /ml), or both EMF and TPA (E+T) for 10, 15, 20, and 30 minutes. The levels of the activated form of MAPK in the cell lists were determined by Western blots as described in Figure 12. The data were normalized by the amount of total protein. The values of the bars (E/C) are the ratios of levels of MAPK activation in experimental samples (E) over those the controls (C). * Indicates significant difference from control. The numbers of experiment for different time points of different treatments are: E (10 min – 3, 15 min – 4, 20 min – 3, 30 min – 3); T (10 min – 3, 15 min – 3, 20 min – 3, 30 min – 3); T+E (10 min – 3, 15 min – 3, 20 min – 3, 30 min – 3).

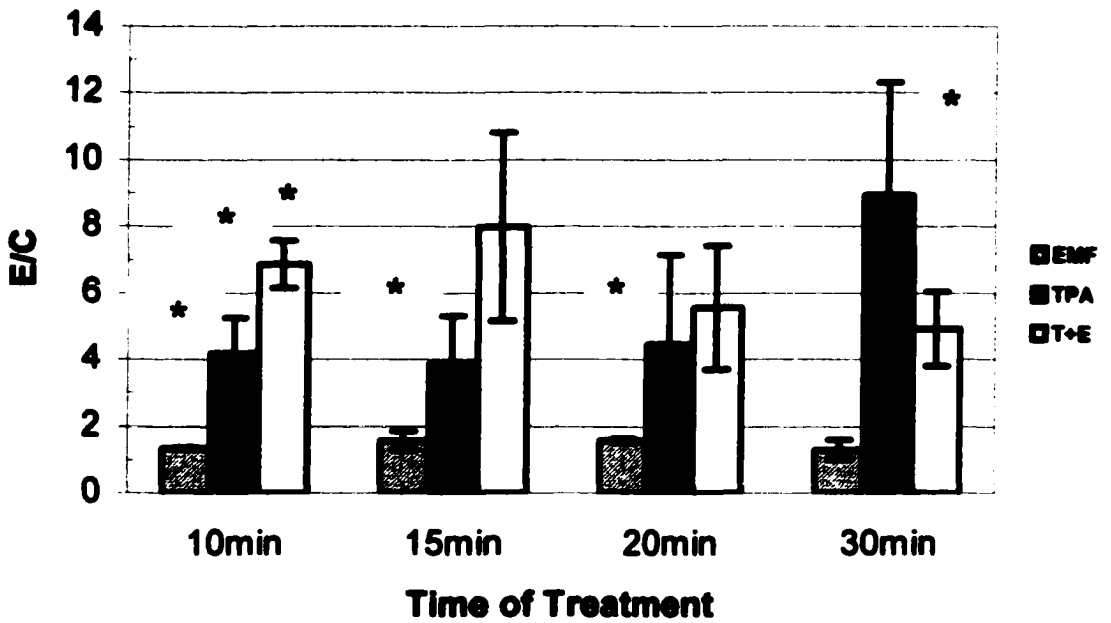

Figure 21. **Effects of EMF exposure, TPA treatment, and EMF exposure in the presence of TPA on MAPK activation in c-Src transfected 3Y1 cells over time.** C-Src transfected 3Y1 cells were exposed to EMF (60 Hz, 1 G), TPA (0.25 ng /ml), or both EMF and TPA (E+T) for 10, 15, 20, and 30 minutes. The levels of the activated form of MAPK in the cell lists were determined by Western blots as described in Figure 12. The data were normalized by the amount of total protein. The values of the bars (E/C) are the ratios of levels of MAPK activation in experimental samples (E) over those the controls (C). * Indicates significant difference from control. The numbers of experiment for different time points of different treatments are: E (10 min – 3, 15 min – 6, 20 min – 3, 30 min – 3); T (10 min – 3, 15 min – 4, 20 min – 3, 30 min – 3); T+E (10 min – 3, 15 min – 3, 20 min – 3, 30 min – 3).

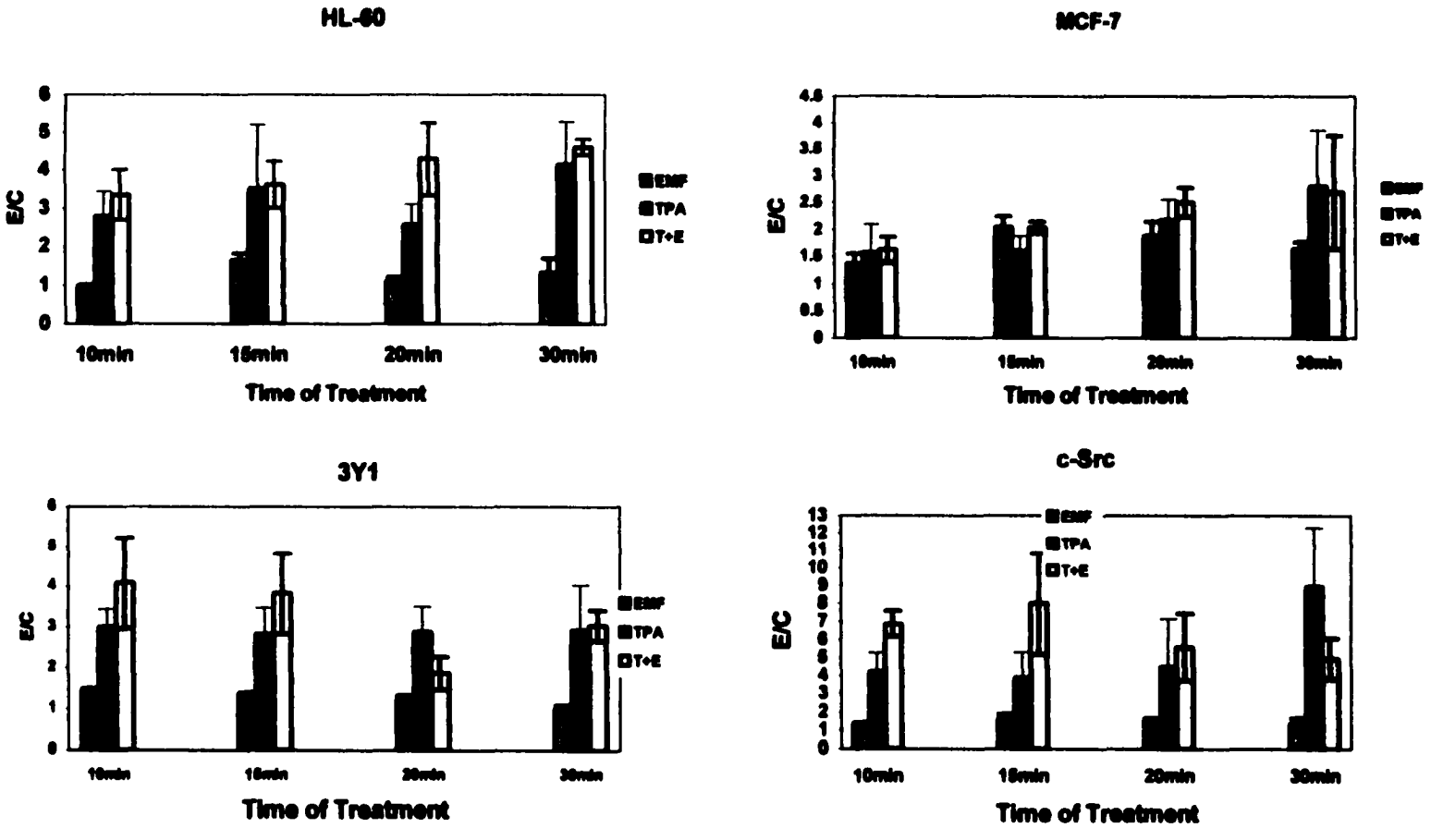

Figure 22. Comparison of the effects of EMF exposure, TPA treatment, and EMF exposure in the presence of TPA on MAPK activation in different cell lines over time. The graph compiles data presented in Figure 19, 20, 21, and 22 to allow a comparison of the modes of reaction of the four cell lines in term of MAPK activation to different treatments (c-Src: 3Y1 cells transfected with c-Src).

observations indicate that EMF and TPA may initiate a similar signal transduction pathway.

The additive effect between EMF and TPA on MAPK induction. A differentiation study of HL-60 cells revealed that an additive effect on cell differentiation exists between field exposure and TPA treatment (Tao and Henderson, 1999). In the present study, the combined effects of EMF and TPA on MAPK activation were explored. HL-60, MCF-7, 3Y1, and c-Src transfected 3Y1 cells were exposed simultaneously to both TPA and EMF to test whether they act synergically or whether their effects on MAPK induction are additive. The results implicated that EMF and TPA could have an additive effect on the activation of MAPK pathway in HL-60 and c-Src transfected cells, in that exposure to EMF in the presence of TPA elicited greater elevation in the level of MAPK activation than exposure to either of them alone, while in 3Y1 and MCF-7 cells such effect was less likely (Table 2 and Figure 22). The differential response of the cell lines to EMF and TPA probably reflects their differences in cellular type, origin, behavior in cultured condition, cell surface receptors, and other intra-cellular modifications.

Effect of PKC inhibitors on EMF induced MAPK activation

It has been proposed that PKC, an upstream activator of MAPK cascade, might be involved in mediating EMF-cell interaction (Wallaczek, 1992; Goodman *et al.*, 1993). This hypothesis has the support of several recent investigations (Monti *et al.*, 1991;

Cell Lines / Duration of Exposure		HL-60	MCF-7	3Y1	C-Src Transfected 3Y1
10 min	(T+E)/E	3.37	1.19	2.82	5.08
	(T+E)/T	1.20	1.04	1.36	1.64
15 min	(T+E)/E	2.21	1.00	2.85	5.06
	(T+E)/T	1.03	1.28	1.35	2.06
20 min	(T+E)/E	3.86	1.33	1.51	3.53
	(T+E)/T	1.68	1.15	0.65	1.25
30 min	(T+E)/E	3.43	1.65	3.06	3.76
	(T+E)/T	1.11	0.96	1.03	0.55

Table 2: **The additive effect between EMF and TPA on MAPK induction in different cell lines.** (T+E)/E or (T+E)/T is the ratio of the E/C value of "T+E" over that of "E" or "T" as showed in Figure 18, 19, 20, and 21.

Uckun *et al.*, 1995; Tuinstra *et al.*, 1998). In previous studies we used PKC-α membrane translocation following TPA induction as a model pathway for interpreting EMF-induced differentiation in HL-60 cells. An increased level of membrane associated PKC-α was detected in cells exposed to EM fields; however, unlike in cells treated with TPA, this increase was not statistically significant.

In this group of experiments, the role of PKC in EMF induced MAPK pathway was investigated indirectly in an inhibition study that focused on two members of the PKC super family: the calcium dependent PKC-α and calcium independent PKC-δ. The effects of three PKC inhibitors, Staurosporine, Gö 6976, and Rottlerin, on EMF or TPA stimulated MAPK activation in HL-60 and MCF-7 cells were tested. Staurosporine is a general inhibitor that inhibits several isoforms of PKC including α and δ; Gö 6976 inhibits PKC-α only; while Rottlerin, with the concentration used in these experiments, inhibits only PKC-δ. Study with these PKC inhibitors of different specificity would provide information as to whether PKC-α, δ, or both are involved in MAPK activation in cells exposed to EMF.

The data reveal that Staurosporine as well as Gö 6976 significantly reduces the level of MAPK activation induced by EMF exposure in both cell lines. On the other hand, Rottlerin seems to have no effect on EMF induced MAPK activation (Figure 23 & 24). The impacts of PKC inhibitors on MAPK activations induced by EMF exposure or TPA treatment are compared in Figure 25 and 26. Staurosporine and Gö 6976 have basically the same effect on MAPK activation resulting from TPA treatment as that from EMF exposure. And the decrease of TPA stimulated MAPK activation in the presence of Rottlerin is not statistically significant.

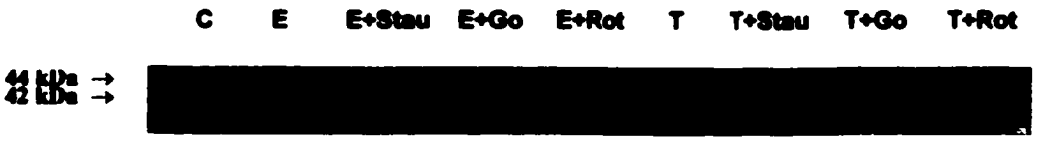

Figure 23. The effects of PKC inhibitors on EMF and TPA induced MAPK activation in MCF-7 cells. MCF-7 cells were exposed to 60 Hz, 1 G EMF (E), treated with 1 ng/ml of TPA (T) or incubated in medium containing Staurosporine (0.2 μM), Gö 6976 (1 μM), or Rottlerin (5 μM) for at least 30 minutes before being exposed to EMF or TPA for 15 minutes (Stau: Staurosporine; Go: Gö 6976; Rot: Rottlerin; C: control). Activated MAPK in the cell lysates was detected as described in Figure 12. The density of the bands indicates level of MAPK activation.

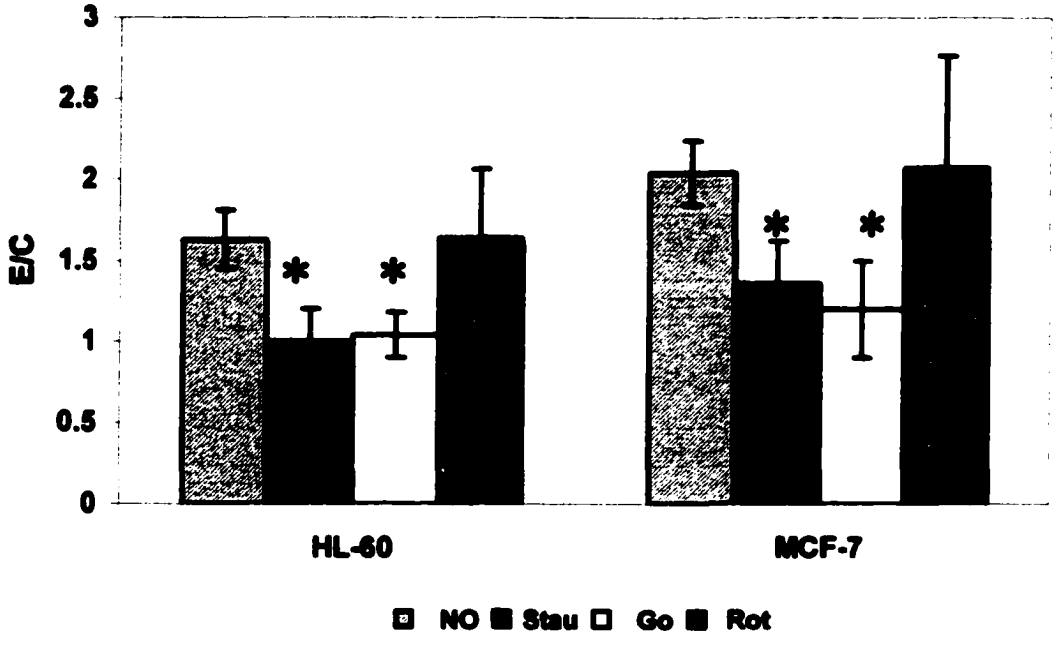

Figure 24. **The effects of PKC inhibitors on EMF induced MAPK activation in HL-60 and MCF-7 cells.** HL-60 or MCF-7 cells were incubated in medium containing Staurosporine (0.2 μM), Gö 6976 (1 μM), or Rottlerin (5 μM) for at least 30 minutes before being exposed to 60 Hz, 1 G EMF for 15 minutes. The levels of the activated form of MAPK in the cell lysates were determined by Western blots as describe in Figure 12. The data were normalized by the amount of total protein. The values of the bars (E/C) are the ratios of levels of MAPK activation in experimental samples (E) over those the controls (C). NO: Without PKC inhibitor; Stau: Staurosporine; Go: Gö 6976; Rot: Rottlerin. * Indicates significant difference from non-inhibition. The numbers of experiment for different inhibitors in different cell lines are: HL-60 cells (No inhibitor – 13, Staurosporine – 5, Gö 6976 – 3, Rottlerin – 4); MCF-7 cells (No inhibitor – 15, Staurosporine – 6, Gö 6976 – 4, Rottlerin – 4).

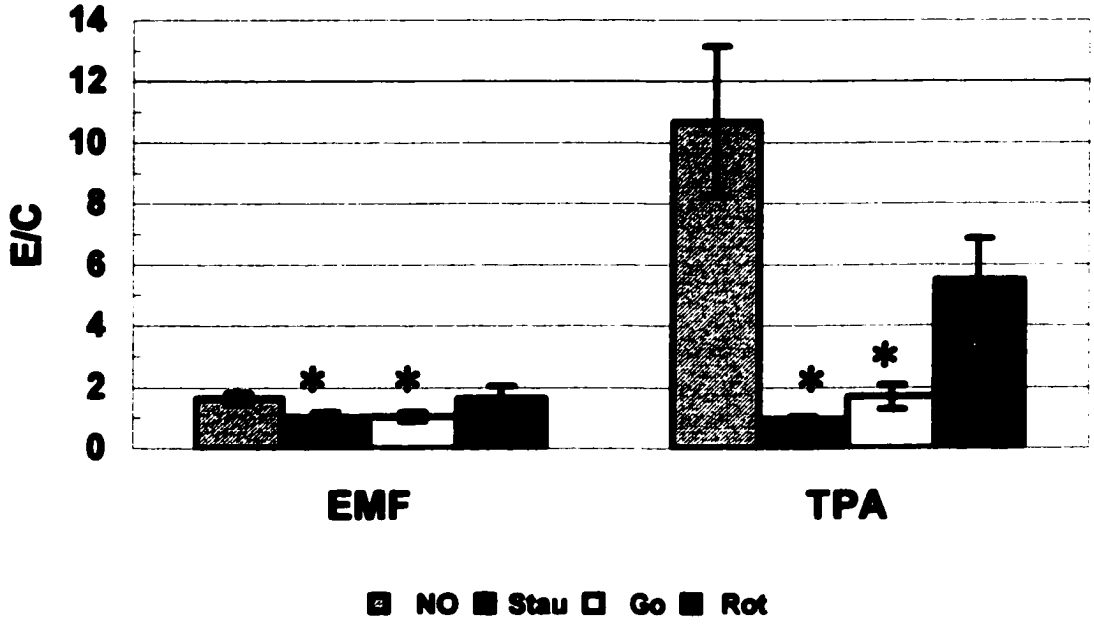

Figure 25. **Comparison of the effects of PKC inhibitors on EMF and TPA induced MAPK activation in HL-60 cells.** HL-60 cells were incubated in medium containing Staurosporine (0.2 μM), Gö 6976 (1 μM), or Rottlerin (5 μM) for at least 30 minutes before being exposed to 60 Hz, 1 G EMF or 1 ng/ml of TPA for 15 minutes. The levels of the activated form of MAPK in the cell lysates were determined by Western blots as describe in Figure 12. The data were normalized by the amount of total protein. The values of the bars (E/C) are the ratios of levels of MAPK activation in experimental samples (E) over those the controls (C). NO: Without PKC inhibitor; Stau: Staurosporine; Go: Gö 6976; Rot: Rottlerin. * Indicates significant difference from non-inhibition. The numbers of experiment for different inhibitors with different treatments are: EMF (No inhibitor – 13, Staurosporine – 5, Gö 6976 – 3, Rottlerin – 4); TPA (No inhibitor – 4, Staurosporine – 4, Gö 6976 – 3, Rottlerin – 4).

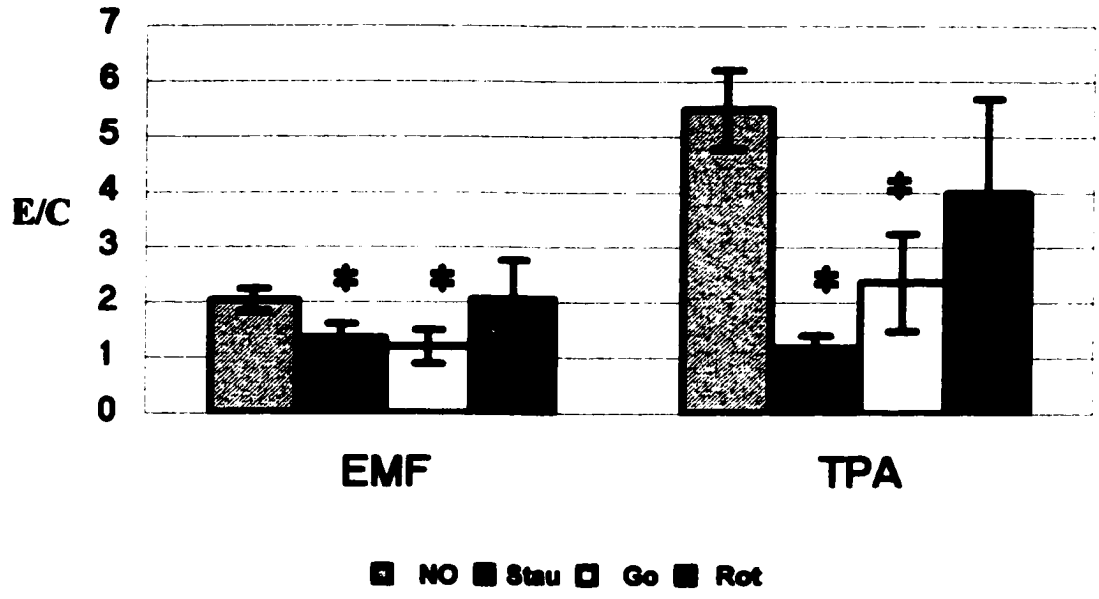

Figure 26. **Comparison of the effects of PKC inhibitors on EMF and TPA induced MAPK activation in MCF-7 cells.** MCF-7 cells were incubated in medium containing Staurosporine (0.2 μM), Gö 6976 (1 μM), or Rottlerin (5 μM) for at least 30 minutes before being exposed to 60 Hz, 1 G EMF or 1 ng/ml of TPA for 15 minutes. The levels of the activated form of MAPK in the cell lysates were determined by Western blots as describe in Figure 12. The data were normalized by the amount of total protein. The values of the bars (E/C) are the ratios of levels of MAPK activation in experimental samples (E) over those the controls (C). NO: Without PKC inhibitor; Stau: Staurosporine; Go: Gö 6976; Rot: Rottlerin. * Indicates significant difference from non-inhibition. The numbers of experiment for different inhibitors with different treatments are: EMF (No inhibitor – 15, Staurosporine – 6, Gö 6976 – 4, Rottlerin – 4); TPA (No inhibitor – 3, Staurosporine – 6, Gö 6976 – 4, Rottlerin – 4).

These results indicate that PKC-α, rather than the calcium independent PKC-δ, is required for the induction of MAPK cascade by EMF or TPA. This implication is in keeping with the speculation that EMF could use Ca^{2+} phospholipid-dependent PKC induction in a way similar to that of TPA or other mitogens.

Estrogens and MAPK Pathway induction in MCF-7 cells

MCF-7 human breast cancer cells are estrogen responsive cells that express both strogen receptor (ER) α and β. Acting as mitogens, estrogens promote proliferation of MCF-7 cells via binding to and subsequently activating the receptors. Tamoxifen, an nonsteroidal antiestrogen, inhibits the growth of MCF-7 cells by competitive binding of ER with estrogen, while the inhibitory effects of melatonin on breast cancer cell proliferation are achieved via down-regulation of ER that is mediated through estrogen-response pathway of the cells (Sutherland *et al.*, 1987; Molis *et al.*, 1994; Molis *et al.*, 1995).

It has been reported that exposure to EMF inhibits the antiproliferative effects of both melatonin and tamoxifen on MCF-7 cells (Blackman *et al.*, 2001; Harland *et al.*, 1999). Since this study shows that MAPK cascade could be stimulated by EMF, it is possible that MAPK pathway is involved the impact of EMF that offsets the action of melatonin and tamoxifen. This possibility leads to the speculation that in addition to TPA induction pathway, the influence of EMF on the growth of MCF-7 cells could be modulated via pathway induced by ligand binding of estrogens to ER as well.

To explore the credibility of this speculation, the effects of beta-estradiol 3-benzoate (EB) on MAPK cascade were tested in MFC-7 cells. The results are presented in Figures 27, 28 and table 3, 4. Although increased levels of Raf-1 phosphorylation on Ser338 and MAPK activation were observed in cells treated with EB, the magnitude of these increments do not depend upon the concentration of EB (In fact no difference was seen in the levels of Raf-1 phosphorylation on Ser338 in cells treated with concentrations of EB ranging from 0.1 nM to 100 nM, while there is a report showing that the impact of EB on cells is dose dependent [Akarasereenont *et al.*, 2000]), and the maximum increase in MAPK activation is only 3- fold. These are in drastic contrast with that observed after TPA treatment, where MAPK activation is dosage dependent and the maximum induction is 50 fold of that in quiescent cells. The presence of estrogens, other hormones, and growth factors in the culture medium may contribute to this phenomenon, considering the complex interrelations between pathways elicited by these mitogens. No decisive conclusion as to whether EB stimulates MAPK pathway in MCF-7 cells used in these experiments could be reached.

	0.1 nM	1 nM	10 nM	100 nM
Number of Experiments	3	5	5	6
E/C: Mean ±SE	1.62 ±0.17	1.72 ±0.51	1.67 ±0.23	1.59 ±0.28
P (Differ from 1)	0.03	0.11	0.02	0.05

Table 3. **Raf-1 phosphorylation on Ser388 in MCF-7 cells treated with beta-estradiol 3-benzoate (EB).** MCF-7 cells were treated with 0.1 nM, 1 nM, 10 nM or 100 nM of EB for 15 minutes. Levels of Raf-1 phosphorylation on Ser338 in the cell lysates was determined by immunoblotting as described in Figure 11. The data were normalized by the amount of total protein. E/C represents the ratio of the level of Raf-1 phosphorylation on Ser338 in experimental sample (E) over that of the control (C).

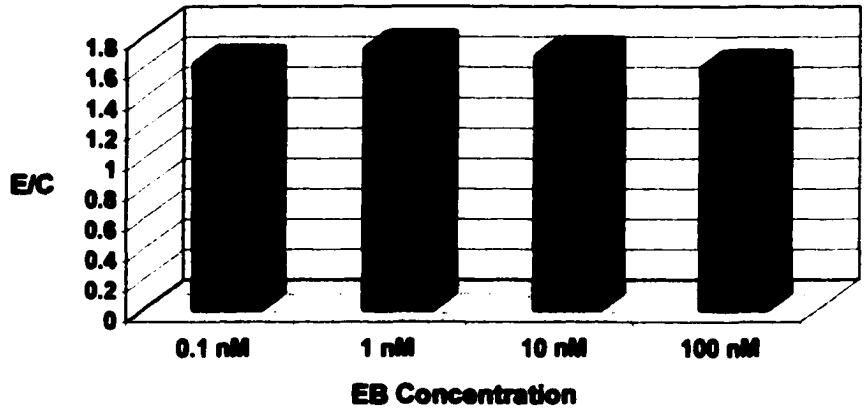

Figure 27. **Raf-1 phosphorylation on Ser388 in MCF-7 cells treated with EB.** MCF-7 cells were treated with 0.1 nM (3), 1 nM (5), 10 nM (5) or 100 nM (6) of EB for 15 minutes (Numbers in parentheses indicate the numbers of experiment done). Levels of Raf-1 phosphorylation on Ser338 in the cell lysates was determined by immunoblotting as described in Figure 11. The data were normalized by the amount of total protein. The values of the bars (E/C) are the ratio of the levels of Raf-1 phosphorylation on Ser338 in experimental samples (E) over those of the controls (C).

	0.1 nM	1 nM	10 nM	100 nM
Number of Experiments	2	3	3	4
E/C: Mean ±SE	2.23 ±1.04	3.1 ±2.04	2.07 ±0.63	4.03 ±1.72
P (Differ from 1)	0.22	0.21	0.12	0.09

Table 4. **Activation state of MAPK in MCF-7 cells treated with EB.** MCF-7 cells were treated with 0.1 nM, 1 nM, 10 nM or 100 nM of EB for 15 minutes. The levels of the activated form of MAPK in the cell lysates were determined by Western blots as describe in Figure 12. The data were normalized by the amount of total protein. E/C represents the ratio of level of MAPK activation in experimental sample (E) over that of the control (C).

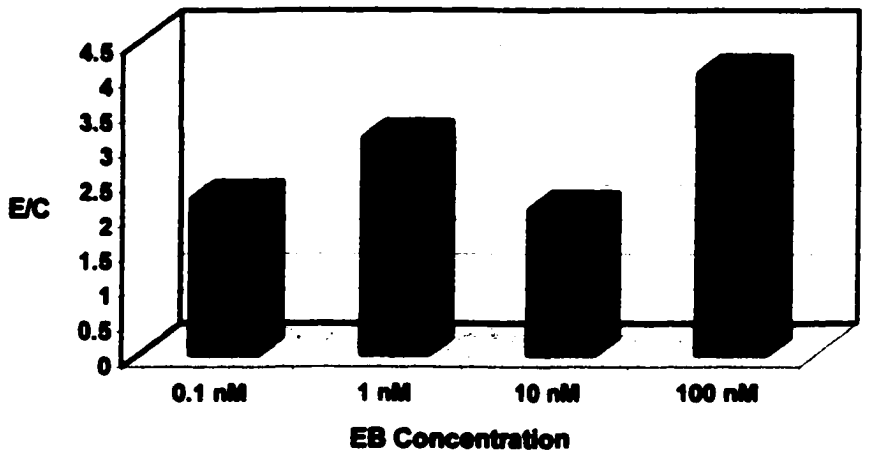

Figure 28. **Activation state of MAPK in MCF-7 cells treated with EB.** MCF-7 cells were treated with 0.1 nM (2), 1 nM (3), 10 nM (3) or 100 nM (4) of EB for 15 minutes (Numbers in parentheses indicate the numbers of experiment done). The levels of the activated form of MAPK in the cell lysates were determined by Western blots as describe in Figure 12. The data were normalized by the amount of total protein. The values of the bars (E/C) are the ratios of levels of MAPK activation in experimental samples (E) over those of the controls (C).

CHAPTER IV

DISCUSSION

A proposed mechanism for EMF-cell interaction that attracted a lot of attention recently is that some unknown event(s) initiated by EM fields at the cell surface is translated by coupling to the cell's signal pathways. The involvement of crucial components of signal transduction systems, such as protein tyrosine kinases (Uckun *et al.*, 1995; Kristupaitis *et al.*, 1998; Dibirdik *et al.*, 1998), PLC-γ2 (Kristupaitis *et al.*, 1998; Dibirdik *et al.*, 1998), PKC (Monti *et al.*, 1991; Tuinstra *et al.*, 1998), and calcium (Wallaczek, 1992; Karabakhtsian *et al.*, 1994; Kim *et al.*, 1998) in cellular responses to EMF have been implicated in several investigations.

A previous study in our lab showed that differentiation of HL-60 cells induced by TPA could also result from exposure to a 60 Hz electromagnetic field (Tao and Henderson, 1999), and the effects of EMF and TPA are additive at lower concentrations of TPA (~250-500 pg/ml). It suggested that EMF and TPA share a common pathway, *i.e.*, one that can be saturated by increasing TPA concentration. Signaling pathway leading to TPA-induced HL-60 cell differentiation is well documented (Nishizuka, 1986; Kharbanda *et al.*, 1994; Marquardt *et al.*, 1994; El-Shemerly *et al.*, 1997), and there are suggestions that it could be affected by EMF exposure as well. Thus, this finding, in the present research provides us with an ideal model system to investigate the signal transduction sequences involved in EMF elicited cellular events, specifically the possible role of MAPK cascade.

The mechanism of TPA action in cells has been studied intensively. PKC acts as a TPA receptor (Niedel *et al.*, 1983). Similar to diacylglycerol (DAG), TPA binds to PKC and mediates a series of signal transduction events leading to cellular responses

(Nishizuka, 1986). The binding of TPA results in the activation of PKC. In contrast to the normal action of DAG, which stimulates PKC only transiently without sustained effect, TPA activates PKC in short-term treatment, while downregulating PKC in prolonged treatment (Rozengurt and Rodriguez-Pena, 1984; Blumberg *et al.*, 1986). Studies show that phorbol ester-induced HL-60 cell differentiation is associated with PKC down-regulation (Solanki *et al.*, 1981; Weinberg *et al.*, 1984).

It is assumed that transducing properties of PKC resides in its ability to phosphorylate proteins (Nishizuka, 1986). In intact cells, phosphorylation may be directed by topological location of PKC following activation (Nishizuka, 1989). TPA causes a rapid translocation (within 10 minutes) of PKC from the cytosolic (soluble) fraction to the particulate (membrane) fraction (Shoji *et al.*, 1986). The membrane translocation of PKC may represent initial events related to the TPA induced terminal differentiation of HL-60 cells along the monocyte/macrophage pathway.

Therefore, an obvious starting point is to explore the role played by PKC in EMF-cell interaction. PKC super family consists of at least 11 distinct isoforms that are divided into three categories on the basis of Ca^{2+} and lipid requirements. The conventional PKCs (cPKC) consist of four members (α, βI, βII, γ) that require both Ca^{2+} and DAG. The novel PKCs (nPKC), include δ, ε, η, and θ, require DAG but are independent of Ca^{2+}. The atypical PKCs (λ, ζ, μ) are independent of Ca^{2+} and insensitive to DAG and phorbol esters.

Our previous studies in HL-60 cells concentrated on the induction of PKC-α by EMF. The results demonstrated that while PKC-α membrane translocation and subsequent down-regulation and depletion was observed after cells were treated with

TPA, a differential response of PKC-α to the field occurs. At most time points, the increase in PKC-α translocation was not statistically significant, and the down-regulation and depletion of total PKC-α in cells under long-term TPA treatment was not seen in cells subjected to prolonged exposure to the field. This was an unexpected result since a time-dependent increase in PKC activity has been reported previously following EMF exposures and is predicted on the basis of our differentiation studies (Monti *et al.*, 1991). Despite the different impacts of EMF and TPA on PKC, this study indicated that the participation of PKC signaling pathway in EMF induced differentiation of HL-60 cells was still likely, although alternative signaling pathways may also exist. The similarities and differences demonstrated in the reaction of PKC-α to EMF and TPA suggested that if PKC mediated EMF-cell interaction at all, the role it played was not exactly the same as the one it played in response to TPA stimulation.

Activation of the MAP kinase cascade (Raf-1 or B-Raf, MEK1 or MEK2, and MAPK/ERK1 or 2) is a pivotal event in mitogen stimulated cell proliferation and differentiation. Two major pathways leading to activation of Raf and ultimately MAP kinase via phosphoryation have been well studied (Jelinek *et al.*, 1996; Crespo *et al.*, 1994; Sozeri *et al.*, 1992; Marais *et al.*, 1996; Bjorkoy *et al.*, 1995; Yamaguchi *et al.*, 1995). PKC and ras are the direct upstream activators in these two pathways. The signal cascade is initiated on cell surface via receptor tyrosine kinase, which either activates PLC leading to PKC activation by producing DAG or IP_3 or induces src family protein kinases. Our data failed to demonstrate definitively a role for PKC in the response of cells to EMF, but we considered the possibility that other pathways leading to

differentiation could be affected by EMF. Whatever pathway is affected by EMF, it includes Raf-1.

It has been shown that there is a correlation between raf-1 hyperphosphorylation and its activation. Raf-1 can be phosphorylation at tyrosine, serine, and threonine residues. Tyrosine phosphorylation appears to be closely associated with the activity of Raf and can be induced by ionizing radiation (Kasid *et al.*, 1996). TPA is shown to phosphorylate raf-1 on both serine and tyrosine residues (Thomas *et al.*, 1992; Kolch *et al.*, 1993). Several reports suggest that activated PKC induced by TPA stimulates the kinase activity of Raf (Sozeri *et al.*, 1992) via phosphorylation of Raf (Kharbanda *et al.*, 1994; Kolch *et at.*, 1993; Marquardt *et al.*, 1994). Kolch *et al.* (1993) showed that PKCα directly phosphorylated Raf-1 on Ser259 and Ser499, but not the tyrosine residues Tyr340 and Tyr341.

Other studies indicated that GTP-binding ras is involved in signal transduction between src family PTK and MAP kinase cascade (Marais *et al.*, 1995, Carol *et al.*, 1994, Crespo *et al.*, 1994). Raf forms a complex with GTP-binding Ras upon mitogen stimulation, which is ensued by its recruitment to the membrane where it is phosphorylated on the tyrosine residues by protein tyrosine kinases (Fabian *et al.*, 1993, Marais *et al.*, 1995). Research by Jelink et al. (1996) suggests that raf-1 activity is phosphorytyrosine dependent. Earlier studies also indicated that Ras was essential for NGF- and TPA-induced tyrosine phosphorylation of MAP kinases, which are downstream effectors of Raf. The activity of MAP kinases in these occasions also seemed to be tyrosine phosphorylation dependent (Thomas *et al.*, 1992). Recent studies

show that phosphorylation at several serine residues, especially Ser338, is crucial for Ras-dependent activation of Raf-1 as well (Diaz *et al.*, 1997; King *et al.*, 1998).

Although there is no evidence to date of direct tyrosine phosphorylation of Raf by PKC, several reports suggest that Ras plays a key role in PKC mediated activation of Raf induced by TPA and EGF. It might function downstream of PKC (Nori *et al.*, 1992, Alexandropoulos *et al.*, 1993, Marais *et al.*, 1996, Marais *et al.*, 1998). Thus, it is highly probable that PKC may participate indirectly in Raf phosphorylation on tyrosine site, for example Tyr340 and Tyr341 in pathway mediated by Src (Fabian *et al.*, 1993).

In the present study, the phosphorylation state of Raf-1 in cells exposed to EMF is examined, starting with the general tyrosine phosphorylation of Raf-1. Tyrosine, rather than serine phosphorylation, was chosen to initiate the studies, because a study by Kasid *et al.* (1996) showed that Raf tyrosine phosphorylation and activation could be induced by ionizing radiation. Though ionizing radiation has a much higher energy than ELF EMF, it still provides a model system to begin our investigation. Technically, the tyrosine phosphorylation level of Raf-1 is easier to determine.

Studies with PKC-α suggests that EMF exposure induces PKC activation within the same time frame as TPA at early time periods. The data of raf-1 tyrosine phosphorylation in HL-60 cells subjected to EMF exposure (1 G, 60 Hz) or TPA (1 ng/ml) treatment presented in this thesis repeat the same trend. There is a correlation between TPA and EMF induced Raf-1 tyrosine phosphorylation relative to time of exposure, leading to the speculation that EMF and TPA induction may use similar route to commonly affect Raf-1 and other effectors downstream. The effect of EMF (or TPA) on tyrosine phosphorylation of Raf-1 was rather small and inconsistent and peaked at about

10 minutes of exposure (30%). This raised the possibility that EMF, as well as TPA, either does not activate Raf-1 solely via tyrosine phosphorylation or some of the phosphorylated tyrosine residues may not be easily accessed to the anti-phosphotyrosine. Raf-1 from TPA treated cells is highly phosphorylated, as indicated by a retardation of Raf-1 in SDS polyacrylamide gel electrophoresis (a sign of hyperphosphorylation), however, this is not reflected in the level of tyrosine phosphorylation of Raf-1 detected in this experiment Thus, we suspected that phosphorylation at serine or threonine residues may play a role as well.

Total serine and threonine phosphorylation of Raf-1 could not be detected with the experimental approach used in this thesis, probably due to the inaccessibility of the phosphorylated sites to the either anti-phosphoserine or threonine. An increased level of phosphorylation on Ser338 of Raf-1 was observed in HL-60 and MCF-7 cells exposed to EMF as well as TPA, which signifies the possible involvement of Ras. Thus, the present study on Raf-1 indicates that either both tyrosine and serine phosphorylation may be required for the activation of the kinase, or there are parallel pathways leading to Raf-1 induction.

By means of phosphorylation, activated Raf turns on MEK, a serine/threonine kinase, which stimulates yet another serine/threonine kinase, MAP kinase, also by phosphorylation. After activation, MAP kinase translocates into the nucleus where it regulates the activities of transcription factors such as Elk-1, c-Myc, c-Jun, and c-Fos via phosphorylation, leading to transcription activation of early response genes such as c-*myc*, c-*jun*, and c-*fos*, resulting in proliferation and differentiation of the cells (Paris and Pouyssegur, 1991; Ainbider *et al.*, 1997; Leppa *et al.*, 1998).

We have shown in this study that MAPK was activated temporarily in HL-60, MCF-7, and 3Y1 (parental and c-Src transfected) cells exposed to low energy EMF (60 Hz, 1 G) to the same extent seen in cells subjected to treatment of low concentration of TPA (0.05-0.6 ng/ml). Human breast cancer cells MCF-7 and rat fibroblast 3Y1 cells were also tested in these experiments in addition to HL-60 cells to extend the investigation into a possible role for the MAPK cascade. This correlation would result in an explanation of other bioeffects of EMF exposure such as elevation of cell proliferation and co-promotion of tumor. The cells studied have different behaviors under cultured conditions. Unlike HL-60 cells that grow in suspension, epithelial MCF-7 cells and fibroblastic 3Y1 cells grow on inert surface. Thus, intercellular communication plays a role in their response to extracellular stimulation. MCF-7 cells are interesting because epidemiological studies indicated a correlation between breast cancer and EMF exposure (Demers *et al.*, 1991). There are also reports that show that EMF exposure inhibits the antiproliferative effects of both melatonin and tamoxifen in MCF-7 cells (Blackman *et al.*, 2001; Harland *et al.*, 1999). TPA-induced cell transformation is a good model to study tumor promotion and its underlying mechanism. Down-regulation of PKC-δ caused by long-term treatment of TPA transforms 3Y1 cells overexpress the non-receptor class protein tyrosine kinase c-Src, but not the parental cells (Lu *et al.*, 1997).

That fact that MAP kinase activation is induced in all the cell lines exposed to the field implies that MAPK is involved in a wide range of cellular responses to EMF exposure, considering the critical role MAPK cascade plays in connecting extracellular stimulation with transcriptional modification in the nucleus. A similar response seen in

cells exposed to EMF or TPA relative to MAPK activation suggests that a same signal transduction pathway may mediate the reaction of cells to both stimuli.

PKC and Ras are the direct upstream activators of Raf in the two major pathways that converge at Raf-1 leading to the ultimate activation of MAPK. Although the two pathways act independently in certain occasions in the induction of MAPK cascade (Arai and Escobedo, 1996; Alexandropoulos *et al.*, 1992), interactions of the two routes exist, embodied mainly by the cross talks between PKC and Ras. Their complicated relationship has been studied extensively.

Morris *et al.* (1989) reported that scrape-loading oncogenic Ras into Swiss 3T3 cells resulted in PKC activation after 5 minutes. No measurable inositol phosphate formation was induced by Ras, suggesting that Ras activates PKC independent of inositol phospholipids. In contrast, there are studies indicating that PKC functions upstream of Ras in TPA initiated mitogenic signaling (Alexandropoulos *et al.*, 1993). Interestingly, a recent study by Marais *et al.* (1998) demonstrated that stimulation of PKC in COS cells caused activation of Ras and formation of Ras-Raf-1 complexes containing active Raf-1. Raf-1mutations that prevent its association with Ras blocked activation of Raf-1 by PKC. However, activation of Raf-1 by PKC was not affected by dominant negative mutant of Ras, indicating that PKC activates Ras by a mechanism distinct from that initiated by activation of receptor tyrosine kinases.

Previous studies in our laboratory found that translocation of PKC-α from cytosol to membrane, where it is activated via phosphorylation, could be induced by TPA, and possibly EMF, in HL-60 cells. A similar, but much weaker and inconsistent response, was seen in cells exposed to a 60 Hz, 1 G field. This makes it difficult to decide whether

PKC plays a role in mediating EMF-elicited differentiation of HL-60 cells. The inhibition study presented in this thesis shows that MAPK activation induced by either TPA or EMF was suppressed by inhibitor targeted calcium dependent PKC-α (Gδ 6976), but not by inhibitor specific for calcium independent PKC-δ (Rottlerin). This indicates that PKC-α rather than PKC-δ is required for the stimulation of MAPK cascade by EMF. It is in keeping with previous findings showing that calcium-dependent PKC is activated by EMF exposure (Monti *et al.*, 1991). However, for lack of direct evidence, whether PKC-α *per se* is activated in HL-60 cells exposed to 60 Hz, 1 G EMF is still inconclusive. Moreover, the possible existence of an alternative or parallel pathway involving Ras functioning upstream of MAP kinase in EMF induced signal transduction process could not be excluded. More work needs to be done to gain insight into the signal sequence that leads to the activation of MAPK cascade, and subsequence transcription modification of early expressed genes such as c-*fos*, c-*myc*, and c-*jun* etc. But whatever the case, PKC, especially α isotype, is most likely involved.

TPA induced signal transduction pathway was served as a model system in our investigation of the signaling sequence involved in EMF-cell interaction. Binding of TPA to PKC leads to activation of PKC in short-term treatment that results in the induction of MAPK cascade. Data from the present study show that EMF exposure could activate MAPK cascade as well, and implicate that the effects of EMF on MAPK pathway is mediated by PKC-α. Detailed comparison of the impacts of TPA or EMF on Raf-1, MAPK suggests that transduction pathway elicited by EMF has considerable similarity with that by TPA. This underlines the possibility that EMF could be a co-factor in tumor formation.

MCF-7 human breast cancer cells are estrogen responsive cells. Naturally, signal transduction initiated by estrogen-receptor binding at the cell surface would be an ideal model to employ in interpretating the cellular response induced by EMF in this cell line. Unfortunately, the sub-line used in our experiment did not give a definitive response to beta-estradiol 3-benzoate (EB) in term of activation of MAPK cascade, probably due to the presence of estrogens, other hormones and growth factors already exist in the culture medium, or the duration (less that 30 minutes) of the treatment (Keshamouni *et al.*, 2002). But the fact that the greatest increase in MAPK activation was seen in MCF-7 cells among all the cell line tested indicates that activation of MAPK pathway contributes to the ability exhibited by EMF in inhibiting the antiproliferative effects of melatonin and tamoxifen in these cells. A recent report suggests a cross-talk between growth factor (MAPK dependent) and estrogen receptor (ER) mediated signaling, *i.e.* activation of one pathway results in the induction of another (Atanaskova *et al.*, 2002). The same relationship could also exist between EMF elicited transduction pathway and that induced by estrogen-receptor binding, and because of this cross-talk, EMF exposure would be able to reverse the effects imposed by tamoxifen via activation of ER in the absent ligand binding and also compensate the down regulation of ER due to the presence of melatonin via increased ER activation, as a result of MAPK induction. Similarly, the relatively strong response of MCF-7 cells to EMF could be viewed as an outcome of this cross-talk as well.

It has been speculated that due to the weak energy associated with ELF EMF, the contribution of the field to tumorogenesis, if any, is likely to be small and indirect. Rather than initiating a malignancy, EMF might promote or co-promote a pre-existing condition.

It is supported by the present study which shows that any change caused by ELF EMF is likely to be small and temporary. Different cell lines or different cells in a population do not all respond to EMF exposure (Tao and Henderson, 1999). Some cell lines or sublines are more sensitive to EMF than others. The same is true within a population of cells and perhaps only a small number of "initiated" cells are susceptible to EMF induction. This may contribute to the often-contradictory results in this area. Thus, sensitive assays must be developed to detect small changes over background noise. The nature of low energy EMF is that its presence is probably perceived by the cell as small, short-lived disturbance (as compared to TPA). Yet, the possibility of malignancy that resulted from an accumulative effect of chronic exposure cannot be totally ignored.

Finally, the present research again demonstrates that TPA induction pathways provide an ideal model for study of EMF-cell interaction. The shared characteristics seen so far in these two pathways are encouraging. Along this path, we expect to learn more about EMF and cell signaling.

CHAPTER IV

REFERENCES

Adams PD, Parker PJ. 1991. TPA-induced activation of MAP kinase. FEBS Lett 290:77-82.

Agarwal S, Corbley MJ, Roberts TM. 1995. Reconstitution of signal transduction from the membrane to the nucleus in a baculovirus expression system: activation of Raf-1 leads to hypermodification of c-jun and c-fos via multiple pathways. Oncogene 11:427-438.

Ahn NG, Seger R, Krebs EG. 1992. The mitogen-activated protein kinase activator. Curr Opin Cell Biol 4:992-999.

Ainbinder E, Bergelson S, Daniel V. 1997. Signaling pathways in the induction of c-fos and c-jun proto-oncogenes by 3-methylcholanthrene. Recept Signal Transduct 7:279-289.

Akarasereenont P, Techatraisak K, Thaworn A, Chotewuttakorn S. 2000. The induction of cyclooxygenase-2 by 17beta-estradiol in endothelial cells is mediated through protein kinase C. Inflamm Res 49:460-465.

Alessi DR, Saito Y, Campbell DG, Cohen P, Sithanandam G, Rapp U, Ashworth A, Marshall CJ, Cowley S. 1994. Identification of the site in MAP kinase kinase I phosphorylated by p74raf-1. EMBO J 13:1610-1619.

Alexandropoulos K, Qureshi SA, Bruder JT, Rapp U, Foster DA. 1992. The induction of Egr-1 expression by v-Fps is via a protein kinase C-independent intracellular signal that is sequentially dependent upon HaRas and Raf-1. Cell Growth Differ 3:731-737.

Alexandropoulos K, Qureshi SA, Foster DA. 1993. Ha-ras functions downstream from protein kinase C in v-Fps-induced gene expression mediated by TPA response elements.

Arai H, Escobedo JA. 1996. Angiotensin II type 1 receptor signals through Raf-1 by a protein kinase C-dependent, Ras-independent mechanism. Mol Pharmacol 50:522-528.

Arnetz BB, Berg M. 1996. Melatonin and adrenocorticotropic hormone levels in video display unit workers during work and leisure. J Occup Environ Med 38:1108-1110.

Atanaskova N, Keshamouni VG, Krueger JS, Schwartz JA, Miller F, Reddy KB. 2002. MAP kinase/estrogen receptor cross-talk enhances estrogen-mediated signaling and tumor growth but does not confer tamoxifen resistance. Oncogene 21:4000-4008.

Balcer-Kubiczek EK, Harrison GH. 1991. Neoplastic transformation of C3H/10T1/2 cells following exposure to 120-Hz modulated 2.45-GHz microwaves and phorbol ester tumor promoter. Radiat Res 126:65-72.

Baris D, Armstrong BG, Deadman J, Thériault G 1996. A mortality study of electrical utility workers in Quebec. Occup Environ Med 53:25.31.

Bjorkoy G, Overvatn A, Diaz-Meco MT, Moscat J, Johansen T. 1995. Evidence for a bifurcation of the mitogenic signaling pathway activated by Ras and phosphatidylcholine-hydrolyzing phospholipase C. J Biol Chem 270:21299-21306.

Blackman CF, Benane SG, House DE. 2001. The influence of 1.2 micro T, 60 Hz magnetic fields on melatonin- and tamoxifen-induced inhibition of MCF-7 cell growth. Bioelectromagnetics 22:122-128.

Blask DE, Hill SM. 1986. Effects of melatonin on cancer: studies on MCF-7 human breast cancer cells in culture. J Neural Transm Suppl 21:433-449.

Blumberg PM, Pastic G, Lacal JC, Warren SB, Aaronson SA. 1986. Loss of mouse fibroblast cell response to phorbol esters restored by micro-injected protein kinase C. Nature 324:375-377.

Blumenthal NC, Ricci J, Breger L, Zychlinsky A, Solomon H, Chen GG, Kuznetsov D, Dorfman R. 1997. Effects of low-intensity AC and/or DC electromagnetic fields on cell attachment and induction of apoptosis. Bioelectromagnetics 18:264-72.

Brendel H, Niehaus M, Lerchl A. 2000. Direct suppressive effects of weak magnetic fields (50 Hz and 16 2/3 Hz) on melatonin synthesis in the pineal gland of Djungarian hamsters (Phodopus sungorus). J Pineal Res 29:228-233.

Broadbent DE, Broadbent MHP, Male JC, Jones MRL 1985. Health of workers exposed to electric fields. Br J Ind Med 42:75-84.

Burch JB, Reif JS, Yost MG, Keefe TJ, Pitrat CA. 1998. Nocturnal excretion of a urinary melatonin metabolite among electric utility workers. Scand J Work Environ Health 24:183-189.

Burch JB, Reif JS, Yost MG, Keefe TJ, Pitrat CA. 1999. Reduced excretion of a melatonin metabolite in workers exposed to 60 Hz magnetic fields. Am J Epidemiol 150:27-36.

Cain CD, Thomas DL, Adey WR. 1993. 60 Hz magnetic field acts as co-promoter in focus formation of C3H/10T1/2 cells. Carcinogenesis 14:955-960.

Campbell-Beachler M, Ishida-Jones T, Haggren W, Phillips JL. 1998. Effect of 60 Hz magnetic field exposure on c-fos expression in stimulated PC12 cells. Mol Cell Biochem 189:107-111.

Carol A, Lange-Carter, Johnson GL. 1994. Ras-dependent growth factor regulation of MEK kinase in PC-12 cells. Science 265, 1458-1461.

Cho MR, Thatte HS, Lee RC, Golan DE. 1994. Induced redistribution of cell surface receptors by alternating current electric fields. FASEB J 8:771-776.

Clejan S, Dotson RS, Ide CF, Beckman BS. 1995. Coordinated effects of electromagnetic field exposure on erythropoietin-induced activities of phosphatidylinositol-phospholipase C and phosphatidylinositol 3-kinase. Cell Biochem Biophys 27:203-225.

Clejan S, Ide C, Walker C, Wolf E, Corb M, Beckman B. 1996. Electromagnetic field induced changes in lipid second messengers. J Lipid Mediat Cell Signal 13:301-324.

Cobb MH, Goldsmith E. 1995. How MAP kinases are regulated. J Biol Chem 270:14843-14846.

Conti P, Reale M, Grilli A, Barbacane RC, Di Luzio S, Di Gioacchino M, De Lutiis MA, Felaco M. 1999. Effect of electromagnetic fields on several CD markers and transcription and expression of CD4. 201:36-48.

Cook MR, Graham C, Cohen HD, Gerkovich MM. 1992. A replication study of human exposure to 60-Hz fields: effects on neurobehavioral measures. Bioelectromagnetics 13:261-285.

Crespo P, Xu N, Daniotti JL, Troppmair J, Rapp UR, Gutkind JS. 1994. Signaling through transforming G protein-coupled receptors in NIH 3T3 cells involves c-Raf activation. Evidence for a protein kinase C-independent pathways. J Biol Chem 269, 21103-21109.

Davanipour Z, Sobel E, Bowman JD, Qian Z, Will AD. 1997. Amyotrophic lateral sclerosis and occupational exposure to electromagnetic fields. Bioelectromagnetics 18:28-35.

Daum G, Eisenmann-Tappe I, Fries H-W, Troppmair J, Rapp UR. 1994. The ins and outs of Raf kinases. Trends Biochem Sci 19:474-480.

Davis RJ. 1995. Transcriptional regulation by MAP kinases. Mol Reprod Dev 42:459-467.

Demers PA, Thomas DB, Rosenblatt KA, Jiminez LM, McCrea Cruuen MG, Satariano W, Austin DF, Isacson P, Greenberg RS, Key C, Koloniel LN, West DW. 1991. Occupational exposure to electromagnetic fields and breast cancer in man. Am J Epidemiol 134:340-347.

Dent P, Reardon DB, Morrison DK, Sturgill TW. 1995. Regulation of Raf-1 and Raf-1 mutants by Ras-dependent and Ras-independent mechanisms in vitro. Mol Cell Biol 15:4125-4135.

Diaz B, Barnard D, Filson A, MacDonald S, King A, Marshall M. 1997. Phosphorylation of Raf-1 serine 338-serine 339 is an essential regulatory event for Ras-dependent activation and biological signaling. Mol Cell Biol 17:4509-4516.

Dibirdik I, Kristupaitis D, Kurosaki T, Tuel-Ahlgren L, Chu A, Pond D, Tuong D, Luben R, Uckun FM. 1998. Stimulation of Src family protein-tyrosine kinases as a proximal and mandatory step for SYK kinase-dependent phospholipase Cγ2 activation in lymphoma B cells exposed to low energy electromagnetic fields. J Biol Chem 273:4035-4039.

Doucet JP, Stephen D, Squinto P, Bazan NG. 1990. *Fos-jun* and the primary genomic response in the nervous system: Possible physiological role and pathophysiological significance. Mol Neurobiology: 27-55.

El-Shemerly MY, Besser D, Nagasawa M, Nagamine Y. 1997. 12-O-Tetradecanoylphorbol-13-acetate activates the Ras/extracellularsignal-regulated kinase (ERK) signaling pathway upstream of SOS involving serinephosphorylation of Shc in NIH3T3 cells. J Biol Chem 272:30599-30602.

Fabian JR, Daar IO, Morrison DK. 1993. Critical tyrosine residues regulate the enzymatic and biological activity of Raf-1 kinase. Mol Cell Biol 13:7170-7179.

Flipo D, Fournier M, Benquet C, Roux P, Le Boulaire C, Pinsky C, LaBella FS, Krzystyniak K. 1998. Increased apoptosis, changes in intracellular Ca2+, and functional alterations in lymphocytes and macrophages after in vitro exposure to static magnetic field. J Toxicol Environ Health A 54:63-76.

Felaco M, Reale M, Grilli A, De Lutiis MA, Barbacane RC, Di Luzio S, Conti P. 1999. Impact of extremely low frequency electromagnetic fields on CD4 expression in peripheral blood mononuclear cells. Mol Cell Biochem 201:49-55.

Feyching M, Ahlbom A. 1993. Magnetic fields and cancer in children residing near Swedish high-voltage power lines. Am J Epidemiol 138:467-481.

Fisch TM, Prywes R, Roeder RG. 1987. C-*fos* sequences necessary for basal expression and induction by epidermal growth factor, 12-O-tetredecanoylphobol-13-acetate, and calcium ionphore. Mol Cell Biol 6:4305-4316.

Fitzsimmons RJ, Strong DD, Mohan S, Baylink DJ. 1992. Low-amplitude, low-frequency electric field stimulated bone cell proliferation may in part be mediated by increased IGF-II release. J Cell Physiol 150:84-89.

Floderus B, Persson T, Stenlund C, Wennberg A, Ost A, Knave B 1993. Occupational exposure to electromagnetic fields in relation to leukemia and brain tumor: a case-control study in Sweden. Cancer Causes Control 4:465-476.

Galvanovskis J, Sandblom J. 1997. Amplification of electromagnetic signals by ion channels. Biophys J 73:3056-3065.

Goodman R, Bassett CAL, Henderson AS. 1983. Pulsing electromagnetic fields induce cellular transcription. Science 220:1283-1285.

Goodman R, Henderson AS. 1986. Sine Waves enhance cellular transcription Bioelectromagnetics 7:23-29.

Goodman R, Abbott J, Henderson AS. 1987. Transcriptional pattern in the X chromosome of *Sciara coprophila* following exposure to magnetic fields. Bioelectromagnetics 8:1-7.

Goodman R, Henderson AS. 1988. Exposure of salivary gland cells to low frequency electromagnetic fields alters polypeptides synthesis. Proc Natl Acad Sci 85:3928-3932.

Goodman R, Henderson AS. 1991. Transcription in cells exposed to extremely low frequency electromagnetic fields: a review. Bioelectrochem Bioenerget 25:335-355.

Goodman R, Chizmandzhev Y, Henderson AS. 1993. Electromagnectic fields and cells. J Cell Biochem 51:436-441.

Graham C, Cook MR, Cohen HD, Gerkovich MM. 1994. Dose response study of human exposure to 60 Hz electric and magnetic fields. Bioelectromagnetics 15:447-463.

Graham C, Cohen HD, Cook MR, Gerkovich MM, Riffle DW. 1992. Human cardiac response to 60-Hz fields: exposure parameters and individual differences. In: Blank M, editor. Electricity and magnetism in biology and medicine. San Francisco: San Francisco Press. P 767-767.

Gupta SK, Gallego C, Johnson GL, Heasley LE. 1992. MAP kinase is constitutively activated in gip2 and src transformed rat 1a fibroblasts. J Biol Chem 267:7987-7990.

Harland JD, Liburdy RP. 1997. Environmental magnetic fields inhibits the antiproliferative action of tamoxifen and melatonin in a human breast cancer cell line. Bioelectromagentics 18:555-562.

Harland J, Engstrom S, Liburdy R. 1999. Evidence for a slow time-scale of interaction for magnetic fields inhibiting tamoxifen's antiproliferative action in human breast cancer cells. Cell Biochem Biophys 31:295-306.

Heidecker G, Kolch W, Morrison DK, Rapp UR. 1992. The role of Raf-1 phosphorylation in signal transduction. Adv Cancer Res 58:53-73.

Hiraki Y, Endo N, Takigawa M, Asada A, Takahashi H, Suzuki F. 1987. Enhanced responsiveness to parathyroid hormone and induction of functional differentiation of cultured rabbit costal chondrocytes by a pulsed electromagnetic field. Biochem Biophys Acta 931:94-100.

Huang W, Alessandrini A, Crews CM, Erikson RL. 1993. Raf-1 forms a stable complex with Mek1 and activates Mek1 by serine phosphorylation. Proc Natl Acad Sci 90:10947-10951.

Hunter T. 1995. Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling. Cell 80:225-236.

Ishido M, Nitta H, Kabuto M. 2001. Magnetic fields (MF) of 50 Hz at 1.2 microT as well as 100 microT cause uncoupling of inhibitory pathways of adenylyl cyclase mediated by melatonin 1a receptor in MF-sensitive MCF-7 cells. Carcinogenesis 22:1043-1048.

Jauchem JR. 1997. Exposure to extremely-low frequency electromagnetic fields and radiofrequency radiation: cardiovascular effects in humans. Int Arch Occup environ Health 70:9-21.

Jelinek T, Dent P, Sturgill TW, Weber MJ. 1996. Ras-induced activation of Raf-1 is dependent on tyrosine phosphorylation.

Jelmert O, Hansteen I-L, Langård S 1994. Chromosome damage in lymphocytes of stainless steel welders related to past and current exposure to manual metal arc welding fumes. Mutat Res 320:223-233.

Johnson MT, Vanscoy-Cornett A, Vesper DN, Swez JA, Chamberlain JK, Seaward MB, Nindl G. 2001. Electromagnetic fields used clinically to improve bone healing also impact lymphocyte proliferation in vitro. Biomed Sci Instrum 37:215-20.

Jonai H, Villanueva MB, Yasuda A. 1996. Cytokine profile of human peripheral blood mononuclear cells exposed to 50 Hz EMF. Ind Health 34:359-68.

Juutilainen J, Stevens RG, Anderson LE, Hansen NH, Kilpelainen M, Kumlin T, Laitinen JT, Sobel E, Wilson BW. 2000. Nocturnal 6-hydroxymelatonin sulfate excretion in female workers exposed to magnetic fields. J Pineal Res 28:97-104.

Kharbanda S, Saleem A, Emoto Y, Stone R, Rapp U, Kufe D. 1994. Activation of Raf-1 and mitogen-activated protein kinases during monocyticdifferentiation of human myeloid leukemia cells. J Biol Chem 14:269: 872-878.

King AJ, Sun H, Diaz B, Barnard D, Miao W, Bagrodia S, Marshall MS. 1998. The protein kinase Pak3 positively regulates Raf-1 activity through phosphorylation of serine 338. Nature 396:180-183.

Lagroye I, Poncy JL. 1998. Influences of 50-Hz magnetic fields and ionizing radiation on c-jun and c-fos oncoproteins. Bioelectromagnetics 19:112-116.

Leppa S, Saffrich R, Ansorge W, Bohmann D. 1998. Differential regulation of c-Jun by ERK and JNK during PC12 cell differentiation. EMBO J 17:4404-4413.

Karabakhtsian R, Broude N, Shalts N, Kolchlaty S, Goodman R, Henderson AS. 1994. Calcium is necessary in the cell response to EM fields. FEBS Lett 349:1-4.

Kasid U, Suy S, Dent P, Ray S, Whiteside TL, Sturgill TW. 1996. Activation of Raf by ionizing radiation. Nature 382:813-816.

Keshamouni VG, Mattingly RR, Reddy KB. 2002. Mechanism of 17-beta-estradiol-induced Erk1/2 activation in breast cancer cells.A role for HER2 AND PKC-delta. J Biol Chem 277:22558-22565.

Kim YV, Conover DL, Lotz WG, Cleay SF. 1998. Electric field-induced changes in agonist-stimulated calcium fluxes of human HL-60 leukemia cells. Bioelectromagnetics 19:366-376.

Kolch W, Heidecker G, Kochs G, Hummel R, Vahidi H, Mischak H, Finkenzeller G, MarméD, Rapp UR. 1993. Protein kinase C activates RAF-1 by direct phosphorylation. Nature 364:249-252.

Kristupaitis D, Dibirdik I, Vassilev A, Mahajan S, Kurosaki T, Chu A, Tuel-Ahlgren L, Tuong D, Pond D, Luben R, Uckun FM. 1998. Electromagnetic field-induced stimulation of Bruton's tyrosine kinase. J Biol Chem 273:12397-12401.

Lee RM, Cobb MH, Blackshear PJ. 1992. Evidence that extracellular signal regulated kinases are the insulin-activated Raf-1 kinase kinases. J Biol Chem 267:1088-1092.

Li CM, Chiang H, Fu YD, Shao BJ, Shi JR, Yao GD. 1999. Effects of 50 Hz magnetic fields on gap junctional intercellular communication. Bioelectromagnetics 20:290-294.

Liboff AR, Williams T Jr, Strong DM, Wistar R Jr. 1984. Time-varying magnetic fields: effect on DNA synthesis. Science 223:818-820.

Liburdy R. 1992. Calcium signaling in lymphocytes and ELF fields: Evidence for an electric field metric and a site of interaction involving the calcium ion channel. FEBS Lett 301:53-59.

Liburdy RP, Callahan DE, Harland J, Dunham E, Sloma TR, Yaswen P. 1993a. Experimental evidence for 60 Hz magnetic fields operating through the signal transduction cascade. Effects on calcium influx and c-MYC mRNA induction. FEBS Lett 22;334:301-308.

Liburdy RP, Sloma TR, Sokolic R, Yaswen P. 1993b. ELF magnetic fields, breast cancer, and melantonin: 60 Hz fields blocks melatonin's oncostatic action on ER+ breast cancer cell proliferation. J Pineal Res 14:89-97.

Lin H, Blank M, Rossol-Haseroth K, Goodman R. 2001. Regulating genes with electromagnetic response elements. J Cell Biochem 81:143-8.

Lindstrom E, Lindstrom P, Berglund A, Mild KH, Lundgren E. 1993. Intracellular calcium oscillations induced in a T-cell line by a weak 50 Hz magnetic field. J Cell Physiol 156:395-398.

Lindstrom E, Berglund A, Mild KH, Lindstrom P, Lundgren E. 1995a. CD45 phosphatase in Jurkat cells is necessary for response to applied ELF magnetic fields. FEBS Lett 370:118-122.

Lindstrom E, Lindstrom P, Berglund A, Lundgren E, Mild KH. 1995b. Intracellular calcium oscillations in a T-cell line after exposure to extremely-low-frequency magnetic fields with variable frequencies and flux densities. Bioelectromagnetics 16:41-47.

Lindstrom E, Still M, Mattsson MO, Mild KH, Luben RA. 2001. ELF magnetic fields initiate protein tyrosine phosphorylation of the T cell receptor complex. Bioelectrochemistry 53:73-78.

Linet MS, Hatch EE, Kleinerman RA, Robinson LL, Kaune WT, Friedman DR, Severson RK, Haines CM, Hartsock CT, Niwa S, Wacholder S, Tarone RE 1997. Residential exposure to magnetic fields and acute lymphoblastic leukemia children. N Eng J Med 337:1-7.

London SJ, Thomas DC, Bowman JD, Sobel E, Cheng TC, Peters JM 1991. Exposure to residential electric and magnetic fields and risk of childhood leukemia. Am J Epidemiol 134:923-937.

Loscher W, Wahnschaffe U, Mevissen M, Lerchl A, Stamm A. 1994. Effects of weak alternating magnetic fields on nocturnal melatonin production and mammary carcinogenesis in rats. Oncology 51:288-295.

Loschinger M, Thumm S, Hammerle H, Rodemann HP. 1998. Stimulation of protein kinase A activity and induced terminal differentiation of human skin fibroblasts in culture by low-frequency electromagnetic fields. Toxicol Lett 96-97:369-376.

Lu Z, Hornia A, Jiang Y, Zang Q, Ohno S, Foster DA. 1997. Tumor promotion by depleting cells of protein kinase C□. Mol Cell Biol. 17:3418-3428

Maniatis T, Fritsch EF, Sambrook J. 1989. Molecular cloning: a laboratory approach. Cold Spring Harbor Laboratory Press, New York.

Marais R, Light Y, Paterson HF, Marshall CJ. 1995. Ras recruits Raf-1 to the plasma membrane for activation by tyrosine phosphorylation. EMBO J 14:3136-3145.

Marais R, Light Y, Mason C, Paterson H, Olson MF, Marshall CJ. 1996. Requirement of Ras-GTP-Raf complexes for activation of Raf-1 by protein kinase C. Science Vol. 280, 109-112

Marais R, Light Y, Mason C, Paterson H, Olson MF, Marshall CJ. 1998. Requirement of Ras-GTP-Raf complexes for activation of Raf-1 by protein kinase C. Science 280:109-112.

Maresh CM, Cook MR, Cohen HD, Graham C, Gunn WS. 1988. Exercise testing in the evaluation of human responses to powerline frequency fields. Aviat Space Environ Med 1139-1145.

Marquardt B, Frith D, Stabel S. 1994. Signalling from TPA to MAP kinase requires protein kinase C, raf and MEK: reconstitution of the signalling pathway in vitro. Oncogene 9:3213-3218.

Marron MT, Goodman EM, Sharpe PT, Greenebaum B. 1988. Low frequency electric and magnetic fields have different effects on the cell surface. FEBS lett 230:13-16.

Mason CS, Springer CJ, Cooper RG, Superti-Furga G, Marshall CJ, Marais R. Serine and tyrosine phosphorylations cooperate in Raf-1, but not B-Raf activation. EMBO J 18:2137-2148.

McDowall ME 1986. Mortality of persons resident in the vicinity of electricity transmission facilities. Br J Cancer 53:271-279.

McLean JR, Stuchly MA, Mitchel RE, Wilkinson D, Yang H, Goddard M, Lecuyer DW, Schunk M, Callary E, Morrison D. 1991. Cancer promotion in a mouse-skin model by a 60-Hz magnetic field: II. Tumor development and immune response. Bioelectromagnetics 12:273-287.

Mevissen M, Lerchl A, Loscher W. 1996. Study on pineal function and DMBA-induced breast cancer formation in rats during exposure to a 100-mG, 50 Hz magnetic field. J Toxicol Environ Health 48:169-185.

Molis TM, Spriggs LL, Jupiter Y, Hill SM. 1995. Melatonin modulation of estrogen-regulated proteins, growth factors, and proto-oncogenes in human breast cancer. J Pineal Res 18:93-103.

Molis TM, Spriggs LL, Hill SM. 1994. Modulation of estrogen receptor mRNA expression by melatonin in MCF-7 human breast cancer cells. Mol Endocrinol 8:1681-1690.

Monti MG, Pernecco L, Morussi MS, Battini R, Zaniol P, Barbiroli P. 1991. Effects of ELF pulse electromagnetic fields on protein kinase C activation process in HL-60 leukemia cells. J. Bioelectricity 10:119-131.

Morris JD, Price B, Lloyd AC, Self AJ, Marshall CJ, Hall A. 1989. Scrape-loading of Swiss 3T3 cells with Ras protein rapidly activates protein kinase C in the absence of phosphoinositide hydrolysis. Oncogene 4:27-31.

Morrison DK, Heidecker G, Rapp UR, Copeland TD. 1993. Identification of the major phosphorylation sites of the Raf-1 kinase. J Biol Chem 268:17309-17316.

Morrison DK. 1995. Mechanisms regulating Raf-1 activity in signal transduction pathways. Mol Reprod Dev 42:507-514.

Niedel JE, Kuhn LJ, Vandenbark GR. 1983. Phorbol diester receptor copurifies with protein kinase C. Proc Natl Acad Sci USA 80:36-41.

Nindl G, Swez JA, Miller JM, Balcavage WX. 1997. Growth stage dependent effects of electromagnetic fields on DNA synthesis of Jurkat cells. FEBS Lett 414:501-6.

Nishizuka Y. 1986. Studies and perspectives of protein kinase C. Science 233:305-312.

Nishizuka Y. 1989. The family of protein kinase C for signal transduction. JAMA 262:1826-1832.

Noda M, Johnson DE, Chiabrera A, Rodan GA. 1987. Effect of electric currents on DNA synthesis in rat osteosarcoma cells: dependence on conditions that influence cell growth. J Orthop Res 5:253-60.

Nordenson I, Mild KH, Jarventaus H, Hirvonen A, Sandstrom M, Wilen J, Blix N, Norppa H 2001. Chromosomal aberrations in peripheral lymphocytes of train engine drivers. Bioelectromagnetics 22:306-15.

Nordenson I, Mild KH, Östman U, Ljungberg H 1988. Chromosomal effects in lymphocytes of 400 kV-substation workers. Radiat Environ Biophys 27:39-47.

Nori M, L'Allemain G, Weber MJ. 1992. Regulation of tetradecanoyl phorbol acetate - induced responses in NIH 3T3 cells by GAP, the GTPase-activating protein associated with $p^{21c\text{-}ras}$. Molecular and Cellular Biology Vol. 12, 936-945

Paris S, Pouyssegur J. 1991. Mitogenic effects of fibroblast growth factors in cultured fibroblasts. Interaction with the G-protein-mediated signaling pathways. Ann N Y Acad Sci 638:139-148.

Perry FS, Reichmanis M, Marino AA, Becker RO 1981. Environmental power frequency magnetic fields and suicide. Health Phys 41:267-277.

Pessina GP, Aldinucci C. 1998. Pulsed electromagnetic fields enhance the induction of cytokines by peripheral blood mononuclear cells challenged with phytohemagglutinin. Bioelectromagnetics 19:445-451.

Pfluger DH, Minder CE. 1996. Effects of exposure to 16.7 Hz magnetic fields on urinary 6-hydroxymelatonin sulfate excretion of Swiss railway workers. J Pineal Res 21:91-100.

Phillips JL, Haggren W, Thomas WJ, Ishida-Jones T, Adey WR. 1992. Magnetic field-induced changes in specific gene transcription. Biochim Biophys Acta 1132:140-144.

Podd JV, Whittington CJ, Barnes J. 1995. Do ELF magnetic fields affect human reaction time? Bioelectromagentics 16:317-23.

Radler-Pohl A, Sachsenmaier C, Gebel S, Auer HP, Bruder JT, Rapp U, Angel P, Rahmsdorf HJ, Herrlich P. 1993. UV-induced activation of AP-1 involves obligatory extranuclear steps including Raf-1 kinase. EMBO J 12:1005-1012.

Rannug A, Ekstrom T, Mild KH, Holmberg B, Gimenez-Conti I, Slaga TJ. 1993. A study on skin tumour formation in mice with 50 Hz magnetic field exposure. Carcinogenesis 14:573-578.

Rao S, Henderson AS. 1996. Regulation of *c-fos* is affected by electromagnetic fields. J Cellular Biochem 63:358-365.

Reichmanis M, Perry FS, Marino AA, Becker RO. 1979. Relation between suicide and the electromagnetic field of overhead power lines. Physiol Chem Phys 11:395-304.

Repacholi MH, Basten A, Gebski V, Noonan D, Finnie J, Harris AW. 1997. Lymphomas in E mu-Pim1 transgenic mice exposed to pulsed 900 MHZ electromagnetic fields. Radiat Res 147:631-640.

Repacholi M, Greenebaum B 1999. Interaction of static and extremely low frequency electric and magnetic fields with living systems: health effects and research needs. Bioelctromagnetics 20:133-160.

Robbins DJ, Zhen E, Owaki H, Vanderbilt CA, Ebert D, Geppert TD, Cobb MH. 1993. Regulation and properties of extracellular signal-regulated protein kinases 1 and 2 in vitro. J Biol Chem 268:5097-5106.

Rodemann HP, Bayreuther K, Pfleiderer G. 1989. The differentiation of normal and transformed human fibroblasts in vitro is influenced by electromagnetic fields. Exp Cell Res. 182:610-21.

Rosen LA, Barber I, Lyle DB. 1998. A 0.5 G, 60 Hz magnetic field suppresses melatonin production in pinealocytes. Bioelectromagnetics 19:123-127.

Rosenthal M, Obe G.1989. Effects of 50-hertz electromagnetic fields on proliferation and on chromosoma alterations in human peripheral lymphocytes untreated or pretreated with chemical mutagens. Mutat Res 210:329-35.

Roy S, Lane A, Yan J, McPherson R, Hancock JF. 1997. Activity of plasma membrane-recruited Raf-1 is regulated by Ras via the Raf zinc finger. J Biol Chem 272:20139-20145.

Rozengurt E, Rodriguez-Pena A. 1984. Disappearance of Ca^{2+}-sensitive, phospholipid-dependent protein kinase activity in phorbol ester-treated 3T3 cells. Biochemical and Biophysical Research Communications 120:1053-1059.

Ruiz Gomez MJ, De la Pena L, Pastor JM, Martinez Morillo M, Gil L. 2001. 25 Hz electromagnetic field exposure has no effect on cell cycle distribution and apoptosis in U-937 and HCA-2/1cch cells. Bioelectrochemistry 53:137-40.

Sahl JD, Kelsh MA, Greenland S 1993. Cohort and nested case-control studies of hematopoietic cancers and brain cancer among electric utility workders. Epidemiology 4:104-114.

Sander R, Brinkmann J, Kuhne B. 1982. Laboratory studies on animals and human beings exposed to 50 Hz electric and magnetic fields. CIGRE, International Congress on Large High Voltage Electric Systems, Paris, CIGRE Paper 36-01.

Savitz DA, Loomis DP 1995. Magnetic field exposure in relation to leukemia and brain cancer mortality among electric utility workers. Am J Epidemiol 141:123-134.

Savitz DA, Boyle CA, Holmgreen P 1994. Prevalence of depression among electrical workers. Am J Ind Med 25:165-176.

Savitz DA, Wachtel H, Barnes FA, John EM, Tvrdik JG 1988. Case control study of childhood cancer and exposure to 60-Hz magnetic fields. Am J Epidemiol 128:21-38.

Seger R, Ahn NG, Posada J, Munar ES, Jensen AM, Cooper JA, Cobb MH, Krebs EG. 1992. Purification and characterization of mitogen-activated protein kinase activator(s) from epidermal growth factor-stimulated A431 cells. J Biol Chem 267:14373-14381.

Selmaoui B, Touitou Y. 1995. Sinusoidal 50-Hz magnetic fields depress rat pineal NAT activity and serum melatonin. Role of duration and intensity of exposure. Life Sci 57:1351-1358.

Selmaoui B, Bogdan A, Auzeby A, Lambrozo J, Touitou Y. 1996. Acute exposure to 50 Hz magnetic field does not affect hematologic or immunologic functions in healthy young men. Bioelectromagnetics 17:364-372.

Selmaoui B, Lambrozo J, Touitou Y. 1997. Endocrine functions in young men exposed for one night to a 50-Hz magnetic field. A circadian study of pituitary, thyroid and adrenocortical hormones. Life Sci 61:473-486.

Seth A, Gonzalez FA, Gupta S, Raden DL, Davis RJ. 1992. Signal transduction within the nucleus by mitogen-activated protein kinase. J Biol Chem 267:24796-24804.

Shoji M, Girard PR, Mazzei GJ, Vogler WR, Kuo JF. 1986. Immunocytochemical evidence for phorbol ester-induced protein kinase C translocation in HL-60 cells. Biochemical and Biophysical Research Communication. 135:1143-1149.

Silny J. 1986. The influence threshold of a time-varying magnetic field in the human organism. In: Bernhardt JH, editor. Biological Effects of Static and Extremely-low-Frequency Magnetic fields. Munich, Germany: MMV Medizin Verlag. P 105-112.

Simko M, Kriehuber R, Weiss DG, Luben RA. 1998. Effects of 50 Hz EMF exposure on micronucleus formation and apoptosis in transformed and nontransformed human cell lines. Bioelectromagnetics 19:85-91.

Sobel E, Davanipour Z 1996. EMF exposure may cause increased production of amyloid beta and eventually lead to Alzheimer's disease. Neurology 47:1594-1600.

Solanki V, Slage TJ, Callaham M, Huberman E. 1981. Down regulation of specific binding of [20-^{3}H] phorbol 12,13-dibutyrate and phorbol ester-induced differentiation of human promyelocytic leukemia cells. Proc Natl Acad Sci USA 78:31722-31725.

Sozeri O, Vollmer K, Liyanage M, Frith D, Kour G, Mark GE, Stabel S. 1992. Activation of the c-Raf protein kinase by protein kinase C Phosphorylation. Oncogene 7, 2259-2262.

Stenlund C, Floderus B 1997. Occupational exposure to magnetic fields in relation to male breast cancer and testicular cancer: a Swedish case-control study. Cancer Causes Control 8:184-191.

Stevens RG. 1987. Electric power use and breast cancer: a hypothesis. Am J Epidemiol 125:294-300.

Stevens RG, Davis S. 1996. The melatonin hypothesis: electric power and breast cancer. Environ Health Perspect 104:135-140.

Stollery BT. 1986. Effects of 50 Hz electric currents on mood and verbal reasoning skills. Br J Ind Med 44:111-118.

Sutherland RL, Watts CK, Hall RE, Ruenitz PC. 1987. Mechanisms of growth inhibition by nonsteroidal antioestrogens in human breast cancer cells. J Steroid Biochem 27:891-897.

Takahashi K, Kaneko I, Date M, Fukada E. 1986. Effect of pulsing electromagnetic fields on DNA synthesis in mammalian cells in culture. Experientia 42:185-6.

Tao Q, Henderson A. 1999. EMF induces differentiation in HL-60 cells. J Cellular Biochem 73:212-217.

Thomas SM, DeMarco M, D'Arcangelo G, Helegoua S, Brugge JS. 1992. Ras is essential for never growth factor - and phorbol ester -induced tyrosine phosphorylation of MAP kinases. Cell vol. 68, 1031-1040.

Thériault G, Goldberg M, Miller AB, Armstrong B, Guenel P, Deadman J, Imbernon E, To T, Chevalier A, Cyr D, Wall C 1994. Cancer risks associated with occupational exposure to magnetic fields among electric utility works in Ontario and Quebec, Canada, and France – 1970-1989. Am J Epidemiol 139:550-572.

Thumm S, Loschinger M, Glock S, Hammerle H, Rodemann HP. 1999. Induction of cAMP-dependent protein kinase A activity in human skin fibroblasts and rat osteoblasts by extremely low-frequency electromagnetic fields. Radiat Environ Biophys 38:195-199.

Toler JC, Shelton WW, Frei MR, Merritt JH, Stedham MA. 1997. Long-term, low-level exposure of mice prone to mammary tumors to 435 MHz radiofrequency radiation. Radiat Res 148:227-234.

Troppnair J, Bruder JT, Munoz H, Lloyd PA, Kyriakis J, Banerjee P, Avruch J, Papp UR 1994. Mitogen-activated protein kinase/extracellular signal-regulated protein kinase activation by oncogenes, serum, and 12-O-tetradecanoylphorbol-13-acetate requires Raf and is necessary for transformation. J Biol Chem 269:7030-7035.

Tuinstra R, Goodman E, Greenebaum B. 1998. Protein kinase C activity following exposure to magnetic field and phorbol ester. Bioelectromagnetics 19:469-476.

Turner BC, Tonks NK, Rapp UR, Reed JC 1993. Interleukin 2 regulates Raf-1 kinase activity through a tyrosine phosphorylation-dependent mechanism in a T-cell line. Proc Natl Acad Sci 90:5544-5548.

Ubeda A, Trillo MA, House DE, Blackman CF. 1995. A 50 Hz magnetic field blocks melatonin-induced enhancement of junctional transfer in normal C3H/10T1/2 cells. Carcinogenesis 16:2945-2949.

Uckun FM, Kurosaki T, Jin J, Jun X, Morgan A, Takata M, Bolen J, Luben R. 1995. Exposure of B-lineage lymphoid cells to low energy electromagnetic fields stimulates Lyn kinase. J Biol Chem 270:27666-27670.

Ueki K, Matsuda S, Tobe K, Gotoh Y, Tamemoto H, Yachi M, Akanuma Y, Yazaki Y, Nishida E, Kadowaki T. 1994. Feedback regulation of mitogen-activated protein kinase kinase kinase activity of c-Raf-1 by insulin and phorbol ester stimulation. J Biol Chem 269:15756-15761.

Valjus J, Norppa H, Järventaus H 1993. Analysis of chromosomal aberrations, sister chromatid exchanges and micronuclei among power linesmen with long-term exposure to 50-Hz electromagnetic fields. Radiat Environ Biophys 32:325-336.

Walleczek J, Liburdy RP. 1990. Nonthermal 60 Hz sinusoidal magnetic-fields exposure enhances $^{45}Ca^{2+}$ uptake in rat thymocytes: dependence on mitogen activation. FEBS Lett 271:157-160.

Walleczek J, Budinger TF. 1992. Pulsed magnetic field effects on calcium signaling in lymphocytes: dependence on cell status and field intensity. FEBS Lett 314:351-355.

Weinberg JB, Misukonis MA, Goodwin BJ. 1984. Human leukemia cell lines with comparable receptor binding characteristics but different phenotypic responses to phorbol diesters. Cancer Research 44:976-980.

Wertheimer N, Leeper E. 1979. Electrical wiring configurations and childhood cancer. Am J Epidemiol 109:273-284.

Wilson BW, Anderson LE, Hilton DI, Phillips RD. 1981. Chronic exposure to 60-Hz electric fields: effects on pineal function in the rat. Bioelectromagnetics 2:371-380.

Wilson BW, Wright CW, Morris JE, Buschbom RL, Brown DP, Miller DL, Sommers-Flannigan R, Anderson LE. 1990. Evidence for an effect of ELF electromagnetic fields on human pineal gland function. J Pineal Res 9:259-269.

Yamaguchi K, Ogita K, Nakamura S, Nishizuka Y. 1995. The protein kinase C isoforms leading to MAP-kinase activation in CHO cells. Biochem Biophys Res Commun 210:639-647.

Yan M, Templeton DJ. 1994. Identification of 2 serine residues of MEK-1 that are differentially phosphorylated during activation by raf and MEK kinase. J Biol Chem 269:19067-19073.

Zang M, Waelde CA, Xiang X, Rana A, Wen R, Luo Z. Microtubule integrity regulates Pak leading to Ras-independent activation of Raf-1. insights into mechanisms of Raf-1 activation. J Biol Chem 276:25157-25165

Zhang J, Zhang F, Ebert D, Cobb MH, Goldsmith EJ. 1995. Activity of the MAP kinase ERK2 is controlled by a flexible surface loop. Structure 3:299-307.

Zhao YL, Johnson PG, Jahreis GP, Hui SW. 1999. Increased DNA synthesis in INIT/10T1/2 cells after exposure to a 60 Hz magnetic field: a magnetic-field or a thermal effect? Radiat Res 151:201-8.

Zwingelberg R, Obe G, Rosenthal M, Mevissen M, Buntenkotter S, Loscher W. 1993. Exposure of rats to a 50-Hz, 30-mT magnetic field influences neither the frequencies of

sister-chromatid exchanges nor proliferation characteristics of cultured peripheral lymphocytes. Mutat Res 302:39-44.