Biochemical Journal Immediate Publication. Published on 25 Apr 2007 as manuscript BJ20061653

BJ2006/1653, Revised

Mechanism of a short-term ERK activation by electromagnetic fields at mobile phone frequency

Joseph Friedman*, Sarah Kraus*, Yirmi Hauptman†, Yoni Schiff†, and Rony Seger*‡

Department of Biological Regulation, The Weizmann Institute of Science, Rehovot 76100, Israel (1) and Gal Safe LTD, Rishon Lezion, Israel 75153 (2)

Short title - ERK activation by mobile phone irradiation

‡ - To whom correspondence should be addressed:

Dr. Rony Seger
Department of Biological Regulation,
The Weizmann Institute of Science
Rehovot 76100, Israel
Tel: 972-8-9343602
Fax: 972-8-9344116
E-mail: rony.seger@weizmann.ac.il

Synopsis

The exposure to non-thermal microwave electromagnetic field generated by mobile phones affects the expression of many proteins. This effect on transcription and protein stability can be mediated by the mitogen-activated protein kinase (MAPK) cascades, which serve as central signaling pathways, and govern essentially all stimulated cellular processes. Indeed, a long-term exposure of cells to mobile phone irradiation results in the activation of p38MAPKs as well as the ERK/MAPKs. Here we studied the immediate effect of irradiation on the MAPK cascades, and found that ERKs, but not stress related MAPKs are rapidly activated in response to various frequencies and intensities. Using signaling inhibitors we delineated the mechanism that is involved in this activation. We found that the first step is mediated in the plasma membrane by NADH oxidase, which rapidly generates reactive oxygen species (ROS). These ROS then directly stimulate matrix metalloproteinases and allow them to cleave and release heparin binding-EGF. This secreted factor, activates EGF receptor, which in turn further activates the ERK cascade. Thus, this study demonstrates for the first time a detailed molecular mechanism by which electromagnetic irradiation by mobile phones induces the activation of the ERK cascade and thereby induces transcription and other cellular processes.

Key words: mobile phone irradiation; ERK; NADH oxidase; reactive oxygen species; Hb-EGF; MMP.

Introduction

The intensive use of cellular phones in the last 20 years had aroused concern about possible health problems that may be caused by the microwave irradiation that is associated with these phones [1]. The current safety standard for the use of mobile phone takes into consideration mainly heating, which is induced by their electromagnetic field. According to these parameters, which are measured by specific absorption rates (SARs, [2]), the amount of energy delivered is too low to trigger biological effects. However, it was shown that electromagnetic fields can affect living tissues by energies that are much lower than those causing changes in tissues' temperature [3]. These temperature-insensitive responses can influence the physiology of cells either in culture [4] or even in organisms [5], but whether this effect can cause pathological changes in higher organisms is still controversial [6].

The response of cells to different types of electromagnetic fields has been demonstrated in various systems and conditions. One of the best-documented responses is the induction of transcription, which can be induced by short exposures to low frequency electric and magnetic fields [7]. In similarity to the above systems, the higher frequency (~900 MHz) electromagnetic irradiation emitted from mobile phones also induces expression of proteins in various cells [4, 8-11]. Among the proteins whose expression is induced by mobile phone irradiation are transcription factors such as c-Jun and c-Fos [10, 12], and heat shock proteins [13], such as Hsp27 [3] and HSP70 [14, 15], but HSP90 levels may be reduced [15]. The elevated expression of these proteins may participate in the induction of various cellular processes that seem to be induced by mobile phones [13], which include replication [4], cell cycle progression [16], and apoptosis [15, 17].

A main mechanism that regulates transcriptional activity in response to extracellular stimuli is the activation of the MAPK cascades. These cascades are a group of signal transduction pathways, which mediate the effects of various stimuli to regulate essentially all stimulated processes including proliferation, differentiation, metabolism and stress response [18-20]. Four canonical MAPK cascades have been identified so far. These are the extracellular signal-regulated kinase 1/2 (ERK1/2; ERKs), JNK1-3 (SAPK1), p38MAPK (SAPK2) and BMK1 (ERK5). Each of the cascades is composed of 3-6 tiers of protein

kinases and their signals are transmitted via a sequential phosphorylation and activation of the protein kinases in each of the tiers. Upon activation, the protein kinases in various tiers phosphorylate and activate a large number of regulatory proteins including a set of transcription factors, which allow the induction of gene expression. Indeed, it was shown that lengthy exposure to mobile phone irradiation can activate the p38MAPK, JNK and ERK cascades [3, 21], although reduction in p38MAPK levels has been reported as well [15]. These changes in MAPKs activity can consequently regulate the physiological response of the exposed cells and organisms, and therefore, are major regulators of the effects of electromagnetic fields at mobile phone frequency. However, in most systems, the ERK cascade is known to be functional within few minutes after activation [20], a time frame that has not been addressed for mobile phone irradiation, and is the subject of the current study.

Using Rat1 and HeLa cells as well as isolated membranes of HeLa cells, we show that ERKs, but not JNKs or p38MAPKs, are rapidly activated in response to mobile phone irradiation at various frequencies and intensities. This activation is mediated by reactive oxygen species (ROS) that are produced upon irradiation by membranal NADH oxidase. The generated ROS then directly activate matrix metalloproteinases (MMPs) to release heparin-binding (Hb)-EGF, which further activate the ERK cascade. Thus, this study delineate, for the first time, a detailed molecular mechanism by which electromagnetic radiation at mobile phone frequency induces short-term MAPK activation.

Experimental procedures

Stimulants, inhibitors, antibodies and miscellaneous reagents. GF109203X, PD098059, SB203580, AG1478, PP2, Anisomycin, MMPI-III, wortmannin and recombinant Hb-EGF were from Calbiochem (San Diego, CA). GM-6001 was from Biomol Research Laboratories (Plymouth Meeting, PA). N-Acetyl cytosine (NAC) and diphenyleneiodonium (DPI) as well as heparin immobilized on agarose were from Sigma Chemicals (St. Louis, Mo). Mouse monoclonal anti-active MAPK (pERK, pJNK and pP38), and rabbit polyclonal anti-ERK, anti-JNK, anti-PKB antibodies were from Sigma (Rehovot, Israel). Anti EGF receptor (EGFR), pEGFR-Tyr1173, and Hb-EGF (C-18), were from Santa Cruz Biotechnology (CA).

Cell culture, stimulation, harvesting and Western blotting. Rat1 and HeLa cells were obtained from the American Type Culture Collection (Rockville, MD) and grown in DMEM supplemented with 10% (v/v) heat-inactivated fetal calf serum (Gibco Laboratories, NY), 1% glutamine and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). Cells were maintained in a humidified atmosphere containing 5% CO_2 at 37^0C. For experiments, the cells were serum-starved in medium containing 0.1% fetal calf serum, 16 hours prior to stimulation. Following treatment, the cells were washed twice with ice-cold phosphate buffered saline and once with buffer A (50 mM β-glycerophosphate (pH 7.3), 1.5 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, and, 0.1 mM sodium orthovanadate). For determination of MAPK phosphorylation the cells were subsequently harvested in ice-cold homogenization buffer (Buffer H: Buffer A and, 1 mM benzamidine, 10μg/ml aprotinin, 10μg/ml leupeptin and, 2μg/ml pepstatin-A) and sonicated (50 watts, 2 X 7 sec), and cell lysates were subjected to centrifugation (20,000Xg, 15 min, 4^0C). The supernatant was assayed for protein content using the Coomassie protein assay (Pierce, Rockford, IL), and equal amounts of proteins were subjected to SDS-PAGE analysis and to Western blotting. The detection was carried on using alkaline phosphatase kit (Promega, WI, USA) or ECL (Amersham, UK) according to the manufacturer's instructions.

Irradiation of cells. Subconfluent cells in 6 cm dishes or suspended membranes were irradiated inside the humidified incubator. We used a frequency generator (signal generator, TGR1040; Thurlby Thandar Instruments; Huntingdon, UK), and amplifier of maximum 1 watt (ERA-3SM; Minicircuit, Seuol, Korea). The generator, located outside of the incubator, was set in the desired power and connected to power amplifier that was connected to a panel antenna that was fixed in the incubator. The emitting antenna was placed in the center of one shelve of the incubator in a distance of 10 cm from each plate. The walls of the incubator were covered with material absorbing irradiation to avoid reflections from the walls, and this created a homogenous irradiating field. A field meter was used to measure the density of irradiation in mW/cm^2 in a particular area of the incubator. After the irradiation, the cells of each plate were washed, harvested and subjected to Western blotting as described above. To

detect Hb-EGF, the medium of the cells was collected, the Hb-EGF was enriched as below, and subjected to Western blotting. The control plates were sham-irradiated.

Enrichment of Hb-EGF from conditioned medium. The enrichment of Hb-EGF from the collected medium of the irradiated cells was done by heparin-agarose beads. Thus, 80 μl beads (50% v/v slurry) were added to 800μl of the collected medium, and incubated for 2 hours under constant shaking. Then, the beads were washed sequentially, once with RIPA buffer, twice with LiCl (0.5M) and twice with buffer A. After the last wash, the beads were boiled in sample buffer (40 μl, 7 min), spun down, and the supernatant containing the Hb-EGF was subjected to Western blot analysis with anti Hb-EGF antibody.

Release of Hb-EGF from plasma membranes. HeLa cells were grown in 10 cm dishes to subconfluency, starved (16 hr 0.1% FCS), washed twice with ice-cold PBS and once with buffer A and scraped into 0.25 M sucrose in buffer H. The cells were then homogenized, and subjected to centrifugation (3000xg, 10 min) to get rid of nuclei. The supernatant was spun down again once in 10,000xg (10 min) to get rid of mitochondria and other organelles and once in high speed (100,000xg, 30 min; Optima ultracentrifuge). The pellet containing purified plasma membranes was suspended in 1 ml phosphate buffered saline to form a suspension. For each point in the irradiation experiment, 100 μl of the suspension were placed in a thin tube and irradiated. After the irradiation, the samples were spun-down again (100,000xg, 30 min) to get rid of membranes, and the supernatant containing Hb-EGF was subjected to Western blotting as above.

NADH oxidases activity in plasma membranes. Plasma membranes of HeLa cells were prepared as described above, and suspended in 0.6 ml reaction buffer containing 250 µM NADH in PBS. The suspended membranes (membranes of $6x10^6$ cells for each reactions) were then either irradiated at 875 MHZ at 37^{o}C for various times or left untreated, after which the samples were transferred to 4^{o}C. To determine NADH oxidase activity [22], the samples were incubated at 37^{o}C for 15 min., and changes of NADH absorption at 340 nM were detected using Ultraspec 2000 spectrometer (Pharmacia Biotech).

Results

Mobile phone irradiation. In this study we examined the molecular mechanisms that sense and transmit mobile-phone-emitted electromagnetic irradiation in tissue culture cells. To do so we irradiated adherent tissue culture cell lines (Rat1 and HeLa) in 6 cm tissue culture plates using various frequencies and intensities. The irradiation system was set up in a normal 37^0C humidified incubator as described under Experimental Procedures. The panel antenna used for irradiation was in an average distance of 10 cm from the tissue culture plates, and the walls of the incubator were covered with irradiation-absorbing material to avoid reflections from the walls, thus creating a homogenous irradiation field. The temperature of the cell's medium was measured throughout the experiment and was found unchanged (Δ<0.05^{o}C) even in the highest intensities used throughout the experiments.

ERKs but not JNKs and p38MAPKs are phosphorylated in response to mobile phone irradiation. A group of enzymes that are known to rapidly respond to extracellular stimuli are the MAPKs, and therefore, their phosphorylation was used to determine the acute effect of the mobile phone irradiation. First, Rat1 and HeLa cells were subjected to the mobile phone irradiation in frequency of 875 MHZ with an intensity of 0.07 mW/cm^2. This intensity is well below the average intensity of a single mobile phone, which is around 0.45 mW/cm^2 in Israel. Under these conditions, ERKs phosphorylation was significantly increased in both Rat1 and HeLa cells (Fig. 1). In the Rat1 cells the phosphorylation peaked at 15 min after irradiation and reduced back to basal level within 30 min, whereas in HeLa cells the peak phosphorylation was 5 min after stimulation and decreased thereafter. Using the same frequency of 875 MHZ we then varied the intensity of irradiation, and found a substantial ERKs phosphorylation already at 0.10 mW/cm^2, which was not much elevated at higher intensities. As expected from many other stimuli [20], the total expression of ERKs was not changed throughout the experiments. In contrast to the significant increase in phospho-ERKs, the phosphorylation of the other MAPKs examined was not significantly changed under any of the conditions used. Thus, in Rat1 cells, irradiation with 0.23 mW/cm^2 had no significant effect on the phosphorylation of JNKs (Fig. 1C), the phosphorylation of p38MAPKα was even slightly reduced (Fig. 1C), and ERK5 phosphorylation was not changed (data not

shown). In HeLa cells the irradiation slightly decreased the phosphorylation of JNK1 (Fig. 1C) and had no effect on the phosphorylation of p38MAPKα (Fig. 1C) and ERK5 (data not shown). The lack of effect of irradiation on the two stress-activated cascades, JNK and p38MAPK, indicates that the irradiation effect on ERKs is not stress-related, and strengthen the notion that the temperature of the system was not changed upon the irradiation.

Kinetics of ERKs phosphorylation. In view of the fact that maximal ERKs phosphorylation was detected already at the relatively low intensity of 0.10 mW/cm^2, we undertook to expend the intensity and time response studies. Thus, Rat1 and HeLa cells were irradiated in a frequency of 875 MHZ with intensities of 0.005, 0.03 and 0.11 mW/cm^2 (Fig. 2). Small increase in phosphorylation (1.4 fold in Rat1 cells and 2 fold in HeLa) was detected already with the lowest intensity; the phosphorylation was higher in 0.03 mW/cm^2 (2.2 folds in Rat1 cells and 2.7 fold in HeLa cells) and in 0.11 mW/cm^2 was very close to the maximal phosphorylation induced by 0.27 mW/cm^2 (3.3 fold in Rat1 cells and 3.6 fold in HeLa cells). In addition to the variation in intensity, we varied the frequency of irradiation in order to cover a wider range of mobile phone usage. Only small differences in ERKs phosphorylation were detected in Rat1 cells irradiated with intensity of 0.07 mW/cm^2 of 800, 875 and 950 MHZ. Interestingly, higher differences in phosphorylation in Rat1 cells were detected for the 46 kDa ERK1b [23], which seems to be highest at 875 MHZ. Thus, it is possible that ERK1b is more sensitive to differences in the frequency of irradiation than ERK1 and ERK2.

Irradiation-induced ERKs phosphorylation is mediated by reactive oxygen species and EGFR. In order to study the possible mechanisms of ERKs phosphorylation in response to mobile phone irradiation, we used various signaling inhibitors including NAC which is a scavenger of ROS, the EGFR inhibitor - AG1478, the PKC inhibitor - GF109203X, the Src inhibitor - PP2, the PI3K inhibitor – wortmannin, and the MEK inhibitor - PD098059. Using 875 MHZ irradiation at intensity of 0.230 mW/cm^2 for both Rat1 and HeLa cells, we found that both NAC and AG1478 inhibit, while GF109203X and PP2 have no effect on the phosphorylation of ERKs in response to mobile phone irradiation (Fig. 3). Wortmannin slightly inhibited ERKs phosphorylation in HeLa but not in Rat1 cells, and as expected, PD098059 inhibited ERKs phosphorylation in both HeLa (Fig. 3B,C) and Rat1 cells (data not

shown). Thus all these results clearly indicate that the irradiation-induced ERKs phosphorylation is mediated by EGFR and ROS, and PI3K may have a partial effect downstream of the receptor in HeLa cells.

Another clue for the mechanism of ERKs phosphorylation in response to mobile phone irradiation came from our finding that 2 minutes irradiation is enough to exert the full effect of ERKs phosphorylation (Fig. 4). Thus, when Rat1 or HeLa cells were irradiated with intensity of 0.21 mW/cm^2 for different times, peak activity was detected 12 min after activation, but similar or even higher phosphorylation was obtained when the cells were irradiated for 2 min and then left in the incubator un-irradiated for additional 10 min. These results indicate that the initial event that is induced by irradiation is done within 2 min, and the signal that is induced by this event further proceed to cause full ERKs activation 10 min later. Alternatively, it is possible that the elevation in activity within the 10 min after shutting the irradiation off is due to inhibition of phosphatases that normally counteract ERKs activation [20]. Such inhibition of phosphatatses could be due to the production of reactive oxygen specifies (ROS) that are known to inhibit phosphatases of the MAPK cascades [24].

Role of MMPs and Hb-EGF in irradiation-induced ERKs phosphorylation. In view of the finding that EGFR mediates ERKs phosphorylation in response to mobile phone irradiation, we undertook to elucidate the mechanism that mediates this process. One of the mechanisms that we studied was the cleavage and release of Hb-EGF by activated MMPs, which is a mechanism involved in the activation of ERKs by some G-protein coupled receptors and stress responses [25]. For this purpose, we irradiated Rat1 and HeLa cells with mobile phone at frequency of 875 MHZ for 15 min in various intensities, and examined the release of Hb-EGF to the surrounding medium. Increase of Hb-EGF release upon mobile phone irradiation was detected in both Rat1 and HeLa cell lines (Fig. 5A), although the amount released from irradiated HeLa cells was much higher than that released from Rat1 cells. Therefore, we continued the study only with the better responders HeLa cells. The time course of Hb-EGF release in these cells was rapid, as a small elevation was detected already 2 min after irradiation. In correlation with ERKs activation, the released Hb-EGF was further increased at later times and especially when the irradiation was for 2 min followed by further

incubation without irradiation for 5 or 10 min (Fig. 5A lower panel). We then used specific signaling inhibitors to identify upstream components that lead to this release. We found that the release of Hb-EGF from the HeLa cells was blocked by NAC as well as by the MMP inhibitor GM-6001 and MMPI-III (Fig. 5B) but not by EGFR inhibitor (data not shown). As expected, EGFR phosphorylation was induced by the irradiation as well (Fig. 5C), and in similarity to Hb-EGF release, this phosphorylation was inhibited by NAC and GM-6001. Finally, NAC (Fig. 3) and the MMP inhibitors GM-6001 and MMPI-III (Fig. 5D) inhibited also ERK phosphorylation upon irradiation of HeLa cells, supporting MMPs involvement upstream of EGFR/ ERKs activation.

To further support the involvement of Hb-EGF in ERKs activation, we undertook to estimate the amount of released Hb-EGF, and verify the ability of such an amount to induce ERK activation. This was done by concentrating the Hb-EGF released to the serum free-medium both before and after irradiation. We found that the concentration procedure was efficient, as less than 15% of the Hb-EGF was lost during this procedure (data not shown). Western blot analysis of the concentrated Hb-EGF as compared with known amounts of commercial purified Hb-EGF revealed that the concentration of released Hb-EGF 5 min after irradiation was about 70 pg/ml increasing to ~220 pg/ml 15 min after irradiation (Fig. 6). These concentrations could indeed induce a significant phosphorylation of ERKs, in similarity to the one induced by the irradiation itself (Fig. 1). Taken together, these data indicate that ERKs phosphorylation upon mobile phone irradiation include production of ROS that activate MMPs, and consequently, release of Hb-EGF to activate EGFR and the ERK cascade.

Irradiation-induced release of Hb-EGF from isolated plasma membranes. Activation of MMPs that leads to Hb-EGF release has previously been reported in several cellular systems [26]. However, it is still unclear whether this activation occurs by a direct ROS effect on membranal enzymes or mediated via ROS-dependent cytoplasmic proteins [27]. We therefore undertook to study whether the release of Hb-EGF in response to mobile phone irradiation can be executed in purified plasma membranes without the presence of cytoplasmic components. To do so, we purified plasma membranes from serum-starved HeLa cells, and

treated them with 875-MHZ mobile phone irradiation (0.300 mW/cm^2). We found that Hb-EGF is released from these membranes 15 and 30 min after irradiation, and this release was significantly reduced when the membranes were preincubated with the inhibitors NAC and GM-6001 (Fig. 7A upper panel). The purity of the membranes was confirmed by probing the isolated membranes with anti-ERKs and anti EGFR antibodies that clearly showed that the membranes were devoid of any significant cytosoplasmic impurities (Fig. 7A lower panels). These results indicate that ROS are activating the MMPs at the plasma membranes, a process that does not seem to require the involvement of cytoplasmic components. A further validation of this notion came when we incubated isolated membranes from serum starved HeLa cells with H_2O_2. We found that also this treatment resulted in a release of Hb-EGF, without significantly changing the amount of EGFR (Fig. 7B). Quantification of these experiments (Fig. 7C) emphasized the significant effects of irradiation, H_2O_2, and the inhibitors, confirming the sequential role of ROS and MMPs in the release of Hb-EGF.

Next we asked what is the component in the plasma membrane that is able to sense the cellular phone irradiation and convert it into ROS production. A membranal enzyme that can generate ROS is NADH oxidase, which can be stimulated by various other cellular stimulations [28, 29]. To examine a possible role of this enzyme in our system, we examined whether membranal NADH oxidase is activated upon irradiation. Indeed, we found that irradiation of isolated membranes for 5 and 10 min significantly elevated NADH oxidation activity (Fig. 8A), indicating that, as reported [30], NADH oxidase does exist in purified HeLa membranes, and its activity is induced upon radiation. NADPH did not serve as a substrate in the isolated membranes (as reported, [30]), and the activity towards NADPH was not enhanced by irradiation (data not shown). To show that the membranal NADH oxidase can induce Hb-EGF release we used DPI, which is a selective inhibitor of the NAD(P)H oxidase [31]. Pretreatment of isolated membranes with DPI significantly reduced the release of Hb-EGF, without affecting the EGFR (Fig. 8B). This inhibitory effect was shown also in intact HeLa cells, where DPI reduced the release of Hb-EGF and phosphorylation of ERKs upon irradiation (Fig. 8C). Therefore, it is likely that the irradiation in HeLa cells is sensed by the membranal NADH oxidase that is activate and induce the rest of the ROS-ERK pathway.

Discussion

In this study we investigated the possible involvement of MAPK signaling in the cellular processes induced by irradiation induced by electromagnetic fields associated with mobile phones. Although activation of ERKs and p38MAPKs in response to mobile phone irradiation has previously been reported in several systems [13], very little is known about the immediate effects of this irradiation and the mechanisms by which it induces the MAPK cascades. We found that the ERK cascade is activated within 5 min and the peak activity is within 10-15 min of irradiation (Figs. 1 and 2). On the other hand, no phosphorylation of JNKs or p38MAPKs was detected in these short times after stimulation, although in similarity to the previous reports [3], longer exposures (4 hours) induced 2-3 fold activation of the stress-related p38MAPKs (data not shown). These results indicate that prior to the stress response, mobile phone irradiation induce an immediate effect in the cytoplasm that activate ERK signaling to further induce transcription of variety of genes [20].

The rapid induction of the ERK cascade raises the question as to what might be the mechanism involved in the perception or sensing of the mobile phone irradiation and how is the signal transmitted to the intracellular signaling machinery. Although mobile phone can induce thermal effects that are thought to activate cellular effects [32], it is unlikely that this is the cause of ERKs activation here. First, it is unlikely that the irradiation intensity of 0.005 for 5 min, which is enough to induce ERKs phosphorytlation can change the medium temperature at all, and indeed, no change in temperature could be detected in the medium even with higher intensities used in our experiments. Second, the fact that the stress related cascades, which are known to be activated by heat or other related stresses are not activated in the time course of our experiments indicate that the activation of ERKs is induced by other mechanisms. This fact, together with the relatively long wavelength of the mobile phone irradiation, necessitated the identification of the unprecedented molecular mechanisms for irradiation-stimulated ERKs phosphorylation, which is the subject of this manuscript.

Our results using signaling inhibitors and isolated plasma membranes suggest that the phosphorylation of ERKs is induced through several steps (Fig. 9). The first step in the processes seems to be irradiation-induced generation of ROS, probably due to activation of

plasma membrane-associated NADH oxidase (Fig. 8). The generated ROS then activate membranal MMPs, which in turn cleave the anchored pro-Hb-EGF and thereby allow shedding of Hb-EGF to the surrounding medium. Finally, the released Hb-EGF binds to EGFR and activates it, to induce ERKs phosphorylation via the Ras-MEK pathway. Thus, production of ROS by mobile phone irradiation, a process, that has already been demonstrated in previous studies [33, 34], is suggested here to be induced by the activation of NADH oxidase, which seems to be the first acceptor of mobile phone irradiation. These events seem to occur within seconds and initiate the later activation of the pathway that leads to ERKs phosphorylation.

Several components of the proposed model (Fig. 9) have been implicated in the activation of the ERK cascade in other cellular systems. Notably, it has been shown that the release of Hb-EGF upon stimulation significantly activates the ERK cascade in various systems [25, 35]. In particular, it is known that binding of ligands to GPCRs induces activation of MMPs such as MMP9 and MMP2 and those cleave the extracellular domain of the membranal pro-Hb-EGF and allow the shedding of the Hb-EGF and its binding to EGFR [36]. In similarity to GPCRs, irradiation and ROS seem to activate intracellular signaling pathways due to stimulation of EGFR [37], which is dependent on the tyrosine autophosphorylation of the latter [38]. Several mechanisms that may induce this irradiation/ROS-dependent activation have been proposed over the past years. These mechanisms included mainly activation of intracellular components such as inhibition of protein Tyr phosphatases [27] and induction of caveolar-induced cytoplasmic events [39]. Another suggested mechanism involves a covalent dimerization of the EGFR that forces its activation as proposed for the effect of peroxynitrite in A431 cells [40]. However, these two mechanisms are unlikely to take place in our system because the purified membranes (Fig. 7) do not contain the cytosolic components, and because no dimerization of EGFR was detected upon irradiation (data not shown). On the other hand, the inhibition of Hb-EGF production in plasma membranes by the ROS scavenger NAC and by MMPs inhibitors (Fig. 7) indicate that the effect of irradiation is mediated by activation of MMPs by generated ROS. This

indicates a direct activation of MMPs by irradiation/ROS, which might act similarly to UVB irradiation-stimulated activation of MMP1 and MMP3 in human dermal fibroblasts [41].

One of the interesting questions raised in our study is the identity of the molecular acceptor of the mobile phone irradiation. Our results (Fig. 8) indicate that this acceptor may be NADH oxidase that seems to be localized in the plasma membrane [29], and convert the irradiation into ROS that subsequently activate MMPs. This mechanism is different from the pathways suggested in other reports in which NADH oxidase expression is induced downstream of the irradiation/ROS and possibly also downstream of ERKs themselves. For example, it was shown that enhancement of MMP2 and MMP9 in cardiac myocytes in response to doxorubicin is mediated by MAPK cascades, which are activated in a redox dependent mechanism [42]. Moreover, it was shown that MMP2 expression, but not activity, is induced in a p47phox containing NADH oxidase [43]. Finally, another example for such a mechanism is the involvement of transactivation of the EGFR in the upregulation of NOX1, a catalytic subunit of NADH oxidase [44]. Therefore, our findings are the first to demonstrate a role of membranal NADH oxidase in directly converting radiation energy into the production of ROS, which in turn initiate intracellular signaling cascades.

An important question that is raised from the activity of NADH oxidase in the isolated membranes that were quite free of cytoplasmic components (Fig. 7) is the identity of the electron donors to the system. Normally, NAD(P)H oxidases use for this purpose NAD(P)H, which is thought to be localized mainly in the cytoplasm and therefore cannot be utilized in our isolated membranes. It is possible that in our isolated plasma membranes hydroquinone is used as the electron donor. This is based on a study by Kishi et al [30] who showed that in HeLa cells there is a transfer of electrons from cytoplasmic NADH (but not NADPH to molecular oxygen via quinones within the lipid bilayar of the plasma membrane. Therefore, the reduced quinones, (hydroquinones), are the natural substrates for the NADH oxidase of HeLa plasma membrane, and are likely to be used in the plasma membranes of HeLa cells used in our experiments. Furthermore, since it was shown that membranal NADH oxidase by itself can initiate intracellular signaling pathways [29], it is possible that this direct activation or the generated ROS can activate PI3K in a EGFR-independent manner.

Since PI3K can activate ERKs via a specific pathway [45], it is possible that the partial effect of the PI3K inhibitor (Fig.3) is a result of an independent mechanism acting in parallel to the EGFR-Ras pathway.

In summary, we show here that ERKs are rapidly activated in response to mobile phone irradiation at various frequencies and intensities. This activation is mediated by ROS that are produced by NADH oxidase upon the irradiation, and directly activate MMPs. In turn, the activated MMPs cleave and release Hb-EGF, which then binds to EGFR, activate it and thereby stimulates the ERK cascade. These studies demonstrate for the first time a detailed molecular mechanism for electromagnetic irradiation-induced MAPK activation.

Acknowledgments

We would like to thank Dr. Daniel Aebersold and other members of the Seger lab for their help in various stages of the study. The study was supported in part by grant form La Foundation Raphael et Regina Levi. Rony Seger is an incumbent of the Yale S. Lewine and Ella Miller Lewine professorial chair for cancer research.

Reference

1 Karger, C. P. (2005) Mobile phones and health: a literature overview. Z Med Phys **15**, 73-85

2 Martinez-Burdalo, M., Martin, A., Anguiano, M. and Villar, R. (2005) On the safety assessment of human exposure in the proximity of cellular communications base-station antennas at 900, 1800 and 2170 MHz. Phys Med Biol **50**, 4125-4137

3 Leszczynski, D., Joenvaara, S., Reivinen, J. and Kuokka, R. (2002) Non-thermal activation of the hsp27/p38MAPK stress pathway by mobile phone radiation in human endothelial cells: molecular mechanism for cancer- and blood-brain barrier-related effects. Differentiation **70**, 120-129

4 Nylund, R. and Leszczynski, D. (2004) Proteomics analysis of human endothelial cell line EA.hy926 after exposure to GSM 900 radiation. Proteomics **4**, 1359-1365

5 Weisbrot, D., Lin, H., Ye, L., Blank, M. and Goodman, R. (2003) Effects of mobile phone radiation on reproduction and development in Drosophila melanogaster. J Cell Biochem **89**, 48-55.

6 Kundi, M. (2004) Mobile phone use and cancer. Occup Environ Med **61**, 560-570, 487

7 Goodman, R. and Blank, M. (2002) Insights into electromagnetic interaction mechanisms. J Cell Physiol **192**, 16-22

8 Nylund, R. and Leszczynski, D. (2006) Mobile phone radiation causes changes in gene and protein expression in human endothelial cell lines and the response seems to be genome- and proteome-dependent. Proteomics **6**, 4769-4780.

9 Remondini, D., Nylund, R., Reivinen, J., Poulletier de Gannes, F., Veyret, B., Lagroye, I., Haro, E., Trillo, M. A., Capri, M., Franceschi, C., Schlatterer, K., Gminski, R., Fitzner, R., Tauber, R., Schuderer, J., Kuster, N., Leszczynski, D., Bersani, F. and Maercker, C. (2006) Gene expression changes in human cells after exposure to mobile phone microwaves. Proteomics **6**, 4745-4754.

10 Chauhan, V., Mariampillai, A., Bellier, P. V., Qutob, S. S., Gajda, G. B., Lemay, E., Thansandote, A. and McNamee, J. P. (2006) Gene expression analysis of a human lymphoblastoma cell line exposed in vitro to an intermittent 1.9 GHz pulse-modulated radiofrequency field. Radiat Res **165**, 424-429

11 Chauhan, V., Mariampillai, A., Gajda, G. B., Thansandote, A. and McNamee, J. P. (2006) Analysis of proto-oncogene and heat-shock protein gene expression in human derived cell-lines exposed in vitro to an intermittent 1.9 GHz pulse-modulated radiofrequency field. Int J Radiat Biol **82**, 347-354

12 Stagg, R. B., Hawel, L. H., 3rd, Pastorian, K., Cain, C., Adey, W. R. and Byus, C. V. (2001) Effect of immobilization and concurrent exposure to a pulse-modulated microwave field on core body temperature, plasma ACTH and corticosteroid, and brain ornithine decarboxylase, Fos and Jun mRNA. Radiat Res **155**, 584-592

13 French, P. W., Penny, R., Laurence, J. A. and McKenzie, D. R. (2001) Mobile phones, heat shock proteins and cancer. Differentiation **67**, 93-97

14 Lin, H., Opler, M., Head, M., Blank, M. and Goodman, R. (1997) Electromagnetic field exposure induces rapid, transitory heat shock factor activation in human cells. J Cell Biochem **66**, 482-488

15 Caraglia, M., Marra, M., Mancinelli, F., D'Ambrosio, G., Massa, R., Giordano, A., Budillon, A., Abbruzzese, A. and Bismuto, E. (2005) Electromagnetic fields at mobile phone frequency induce apoptosis and inactivation of the multi-chaperone complex in human epidermoid cancer cells. J Cell Physiol **204**, 539-548

16 Capri, M., Scarcella, E., Fumelli, C., Bianchi, E., Salvioli, S., Mesirca, P., Agostini, C., Antolini, A., Schiavoni, A., Castellani, G., Bersani, F. and Franceschi, C. (2004) In vitro exposure of human lymphocytes to 900 MHz CW and GSM modulated radiofrequency: studies of proliferation, apoptosis and mitochondrial membrane potential. Radiat Res **162**, 211-218

17 Hook, G. J., Zhang, P., Lagroye, I., Li, L., Higashikubo, R., Moros, E. G., Straube, W. L., Pickard, W. F., Baty, J. D. and Roti Roti, J. L. (2004) Measurement of DNA damage and apoptosis in Molt-4 cells after in vitro exposure to radiofrequency radiation. Radiat Res **161**, 193-200

18 Rubinfeld, H. and Seger, R. (2005) The ERK cascade: a prototype of MAPK signaling. Mol Biotechnol **31**, 151-174

19 Seger, R. and Krebs, E. G. (1995) The MAPK signaling cascade. Faseb J **9**, 726-735

20 Yoon, S. and Seger, R. (2006) The extracellular signal-regulated kinase: multiple substrates regulate diverse cellular functions. Growth Factors **24**, 21-44

21 Jin, M., Blank, M. and Goodman, R. (2000) ERK1/2 phosphorylation, induced by electromagnetic fields, diminishes during neoplastic transformation. J Cell Biochem **78**, 371-379

22 Miller, F. J., Jr. and Griendling, K. K. (2002) Functional evaluation of nonphagocytic NAD(P)H oxidases. Methods Enzymol **353**, 220-233

23 Yung, Y., Yao, Z., Hanoch, T. and Seger, R. (2000) ERK1b, a 46-kDa ERK isoform that is differentially regulated by MEK. J Biol Chem **275**, 15799-15808

24 Lee, K. and Esselman, W. J. (2002) Inhibition of PTPs by H(2)O(2) regulates the activation of distinct MAPK pathways. Free Radic Biol Med **33**, 1121-1132

25 Naor, Z., Benard, O. and Seger, R. (2000) Activation of MAPK cascades by G-protein-coupled receptors: the case of gonadotropin-releasing hormone receptor. Trends Endocrinol Metab **11**, 91-99

26 Fischer, O. M., Hart, S., Gschwind, A. and Ullrich, A. (2003) EGFR signal transactivation in cancer cells. Biochem Soc Trans **31**, 1203-1208

27 Chen, C. H., Cheng, T. H., Lin, H., Shih, N. L., Chen, Y. L., Chen, Y. S., Cheng, C. F., Lian, W. S., Meng, T. C., Chiu, W. T. and Chen, J. J. (2006) Reactive oxygen species generation is involved in epidermal growth factor receptor transactivation through the transient oxidization of Src homology 2-containing tyrosine phosphatase in endothelin-1 signaling pathway in rat cardiac fibroblasts. Mol Pharmacol **69**, 1347-1355

28 del Castillo-Olivares, A., Nunez de Castro, I. and Medina, M. A. (2000) Dual role of plasma membrane electron transport systems in defense. Crit Rev Biochem Mol Biol **35**, 197-220

29 Ushio-Fukai, M. (2006) Localizing NADH Oxidase–Derived ROS. Science STKE www.stke.org/cgi/content/full/2006/349/re8

30 Kishi, T., Morre, D. M. and Morre, D. J. (1999) The plasma membrane NADH oxidase of HeLa cells has hydroquinone oxidase activity. Biochim Biophys Acta **1412**, 66-77

31 Cross, A. R. and Jones, O. T. (1986) The effect of the inhibitor diphenylene iodonium on the superoxide-generating system of neutrophils. Specific labelling of a component polypeptide of the oxidase. Biochem J **237**, 111-116

32 Miyakoshi, J., Takemasa, K., Takashima, Y., Ding, G. R., Hirose, H. and Koyama, S. (2005) Effects of exposure to a 1950 MHz radio frequency field on expression of Hsp70 and Hsp27 in human glioma cells. Bioelectromagnetics **26**, 251-257

33 Zmyslony, M., Politanski, P., Rajkowska, E., Szymczak, W. and Jajte, J. (2004) Acute exposure to 930 MHz CW electromagnetic radiation in vitro affects reactive oxygen species level in rat lymphocytes treated by iron ions. Bioelectromagnetics **25**, 324-328

34 Irmak, M. K., Fadillioglu, E., Gulec, M., Erdogan, H., Yagmurca, M. and Akyol, O. (2002) Effects of electromagnetic radiation from a cellular telephone on the oxidant and antioxidant levels in rabbits. Cell Biochem Funct **20**, 279-283

35 Carpenter, G. (2000) EGF receptor transactivation mediated by the proteolytic production of EGF-like agonists. Sci STKE **2000**, PE1

36 Prenzel, N., Zwick, E., Daub, H., Leserer, M., Abraham, R., Wallasch, C. and Ullrich, A. (1999) EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. Nature **402**, 884-888

37 Dent, P., Yacoub, A., Contessa, J., Caron, R., Amorino, G., Valerie, K., Hagan, M. P., Grant, S. and Schmidt-Ullrich, R. (2003) Stress and radiation-induced activation of multiple intracellular signaling pathways. Radiat Res **159**, 283-300

38 Schmidt-Ullrich, R. K., Mikkelsen, R. B., Dent, P., Todd, D. G., Valerie, K., Kavanagh, B. D., Contessa, J. N., Rorrer, W. K. and Chen, P. B. (1997) Radiation-induced proliferation of the human A431 squamous carcinoma cells is dependent on EGFR tyrosine phosphorylation. Oncogene **15**, 1191-1197

39 Wang, Q., Turlington, A., Heo, S., Blanco, A., Tian, J., Xie, Z., Yan, B. and Wan, Y. (2005) Extracellular matrix activity and caveolae events contribute to cell surface receptor activation that leads to MAP kinase activation in response to UV irradiation in cultured human keratinocytes. Int J Mol Med **15**, 633-640

40 van der Vliet, A., Hristova, M., Cross, C. E., Eiserich, J. P. and Goldkorn, T. (1998) Peroxynitrite induces covalent dimerization of epidermal growth factor receptors in A431 epidermoid carcinoma cells. J Biol Chem **273**, 31860-31866

41 Brenneisen, P., Sies, H. and Scharffetter-Kochanek, K. (2002) Ultraviolet-B irradiation and matrix metalloproteinases: from induction via signaling to initial events. Ann N Y Acad Sci **973**, 31-43

42 Ichihara, S., Noda, A., Nagata, K., Obata, K., Xu, J., Ichihara, G., Oikawa, S., Kawanishi, S., Yamada, Y. and Yokota, M. (2006) Pravastatin increases survival and suppresses an increase in myocardial matrix metalloproteinase activity in a rat model of heart failure. Cardiovasc Res **69**, 726-735

43 Grote, K., Ortmann, M., Salguero, G., Doerries, C., Landmesser, U., Luchtefeld, M., Brandes, R. P., Gwinner, W., Tschernig, T., Brabant, E. G., Klos, A., Schaefer, A., Drexler, H. and Schieffer, B. (2006) Critical role for p47(phox) in renin-angiotensin system activation and blood pressure regulation. Cardiovasc Res **71**, 596-605

44 Fan, C., Katsuyama, M., Nishinaka, T. and Yabe-Nishimura, C. (2005) Transactivation of the EGF receptor and a PI3 kinase-ATF-1 pathway is involved in the upregulation of NOX1, a catalytic subunit of NADH oxidase. FEBS Lett **579**, 1301-1305

45 Chaudhary, A., King, W. G., Mattaliano, M. D., Frost, J. A., Diaz, B., Morrison, D. K., Cobb, M. H., Marshall, M. S. and Brugge, J. S. (2000) Phosphatidylinositol 3-kinase regulates Raf1 through Pak phosphorylation of serine 338. Curr Biol **10**, 551-554

Legends to Figures

Fig. 1 - Mobile phone irradiation induces ERKs, but not JNKs or p38MAPKs, phosphorylation. (A) Serum-starved Rat1 and HeLa cells were irradiated with frequency generator at 875 MHZ with intensity of 0.07 mW/cm^2 for the indicated times. After irradiation the cells were harvested and subjected to Western blot analysis with anti phospho ERKs (pERK) and anti general ERKs (gERK). (B) Serum-starved Rat1 and HeLa cells were irradiated at 875 MHZ with intensities of 0.1, 0.2, 0.31 mW/cm^2 for 10 minutes. ERKs phosphorylation was monitored as above. (C) Serum-starved Rat1 and HeLa cells were irradiated at 875 MHZ with intensity of 0.230 mW/cm^2 for the indicated times. For positive control, serum-starved HeLa cells were also treated anisomycin (1 μg/ml, 15min), after which the cells were washed and harvested as above. JNKs and p38MAPKs phosphorylation was detected with the indicated antibodies.

Fig. 2 - Kinetics of ERKs phosphorylation upon mobile phone irradiation. (A) Serum-starved Rat1 cells were irradiated with frequency generator at 875 MHZ with intensities of 0.005, 0.029 and 0.110 and 0.310 mW/cm^2 for the indicated times. ERKs phosphorylation was analyzed as described above. The results were quantified by densitometry, and the average and standard errors of three experiments are shown in the left panel. (B) Serum-starved HeLa cells were irradiated with at 875 MHZ at intensities of 0.005, 0.03, 0.11 mW/cm^2 and 0.310 mW/cm^2. ERKs phosphorylation was followed as described in A. (C) Serum-starved Rat1 cells were irradiated at 800, 875 and 950 MHZ with intensity of 0.070 mW.cm^2 for the indicated times. ERKs phosphorylation was followed as described in A.

Fig. 3 – Use of inhibitors to identify mediators of irradiation-induced ERKs phosphorylation. (A) Serum-Starved Rat1 cells were incubated for 20 min with the following inhibitors: 2.5 mM NAC, 10 μM AG1478, 3 mM GF109203X, 5μM PP2 250 nM wormannin (Wor) or left untreated as a control (Con). After incubation the cells were either irradiated with frequency generator at 875 MHZ with intensity of 0.210 mW/cm^2 for 10 min (+) or left untreated (-). ERKs phosphorylation was detected as described above. (B) Serum-

Starved HeLa cells were incubated for 20 min with the following inhibitors: 10 μM AG1478 3 mM GF109203X, 25 μM PD98059, 250 nM wortmannin (Wor) 2.5 mM NAC, and 5μM PP2 or left untreated as a control (Con). After incubation the cells were either irradiated at 875 MHZ with intensity of 0.210 mW/CM^2 for 10 min (+) or left untreated (-). ERKs phosphorylation was detected as described above. (C) Quantification of the inhibition experiment. Average and standard errors of 2 or 3 experiments are presented.

Fig. 4 - Continuous effect of irradiation on ERKs phosphorylation. Serum-starved Rat1(A) or HeLa (B) cells were irradiated with frequency generator of 875 MHZ at intensity of 0.17 mW/cm^2 for 2 min, 12 min or were irradiated for 2 min and then left in the incubator without irradiation for additional 5 or 10 min (2+5, 2+10). ERKs phosphorylation was detected as described above.

Fig. 5 - Involvement of Hb-EGF, MMPs and ROS in irradiation induced ERKs phosphorylation. (A) Serum-starved Rat1 (upper panel) and HeLa (lower panel) cells were irradiated with frequency generator at 875 MHZ with intensities of 0.04, 0.09, 0.17 and 0.27 mW/cm^2 for 10 min. For time course determination, serum starved HeLa cells were irradiated at 875 MHZ, 0.31 mW/cm2 for the indicated times (third panel). After stimulation, the starvation medium was collected, Hb-EGF was enriched using heparin beads (as described under Experimental Procedures), and subjected to a Western blot analysis with anti Hb-EGF antibody. (B) Serum-starved HeLa cells were incubated for 20 min with NAC (2.5 mM, upper panel), 0.5 μM GM-6001 and 0.4 μM MMPI-III (lower panel) or left untreated as a control. Then the cells were irradiated at 875 MHZ with the indicated intensities for 10 min. The release of Hb-EGF was detected as in A. (C) Serum-starved HeLa cells were incubated with NAC (2.5 mM, 20 min) GM-6001 (0.5 μM, 20 min) or left untreated. One plate from each treatment was irradiated (875 MHZ, 5 min, 0.21 mW/cm^2), while the other was left un-touched, and then cells were harvested in RIPA buffer and subjected to Western blot analysis with anti pEGFR or anti EGFR as indicated. (D) Serum-starved HeLa cells were treated with GM-6001 (0.5 μM, 20 min), MMPI (0.4 μM) or left untreated. Then the cells were irradiated

at 875 MHZ with intensity of 0.25 or 0.22 mW/cm^2 as indicated. ERKs phosphorylation was detected as described above.

Fig. 6 – The amount Hb-EGF released by irradiation is sufficient to induce ERK activation. (A) Serum starved HeLa cells were irradiated with frequency generator of 875 MHZ at intensity of 0.344 mW/Cm^2 for 5, 10, and 15 minutes. Hb-EGF was enriched using heparin and subjected to a western blot analysis together with known concentrations of Hb-EGF (100, 200 pg). (B) Low concentrations of Hb-EGF (50,100 and 200 pg/ml) were used to stimulate ERK in serum starved HeLa cells. After stimulation for 2-15 min, the cells were harvested and subjected to a Western blot analysis with anti pERK and anti ERK antibodies.

Fig. 7 - Irradiation induces release of Hb-EGF from isolated plasma membranes. (A) Plasma membranes of serum-starved HeLa cells were isolated as described under Experimental procedures. The membranes (5 μl of net membranes dissolved in 100 μl PBS in each condition) were incubated with NAC (2.5 mM), GM-6001 (0.5 μM) or left untreated for 15 min. Then the membranes were irradiated with frequency generator at 875 MHZ at intensity of 0.200 mW/cm^2 for the indicated times. The membranes were then subjected to a Western blot analysis with anti Hb-EGF antibody (upper panel). The membranes, as well as total cell extract (Total) were also analyzed with anti EGFR and ERKs antibodies (second and third panels). (B) Isolated plasma membranes from serum-starved HeLa cells prepared as above were incubated with 100 and 200 μM H_2O_2 for the indicated times. The amount of Hb-EGF and EGFR was determined with the proper antibodies. (C) Quantification (average and error bars) of the results of three distinct experiments.

Fig. 8 – NADH oxidase is involved in irradiation-induced release of Hb-EGF and phosphorylation of ERKs. (A) Plasma membranes of serum-starved HeLa cells were isolated as described under Experimental procedures. The membranes (5 μl of net membranes dissolved in 600 μl of buffer containing 250 μM NADH in PBS) were irradiated with frequency generator at 875 MHZ at intensity of 0.240 mW/cm^2 for the indicated times.

NADH oxidase activity was determined as described under experimental Procedures. The results are average and standard errors of three distinct experiments. (B) Plasma membranes of serum-starved HeLa cells were either incubated with 12 μm DPI (15 min) or left untreated as indicated. Then the membranes were irradiated with frequency generator at 875 MHZ with an intensity of 0.200 mW/cm^2 for the indicated times. The amount of Hb-EGF and EGFR was analyzed by Western blots with the indicated antibodies. (C) Serum-starved HeLa cells were incubated with 12 μM of DPI for 30 min (+) or left untreated as control (-). The cells were then irradiated at 875 MHZ with intensity of 0.200 mW/cm^2 for 10 min. The amount of released Hb-EGF and phosphorylated ERKs was determined with the indicated antibodies as described in Figs.1 and 5.

Fig. 9 – Schematic representation of the proposed mechanism that mediates ERKs phosphorylation upon mobile phone irradiation. This activation is mediated by irradiation-induced activation of NADH oxidase that generates ROS at the plasma membranes. The ROS then directly activate MMPs, to cleave and release Hb-EGF, which further binds EGFR and activates the ERK cascade.

Biochemical Journal Immediate Publication. Published on 25 Apr 2007 as manuscript BJ20061653

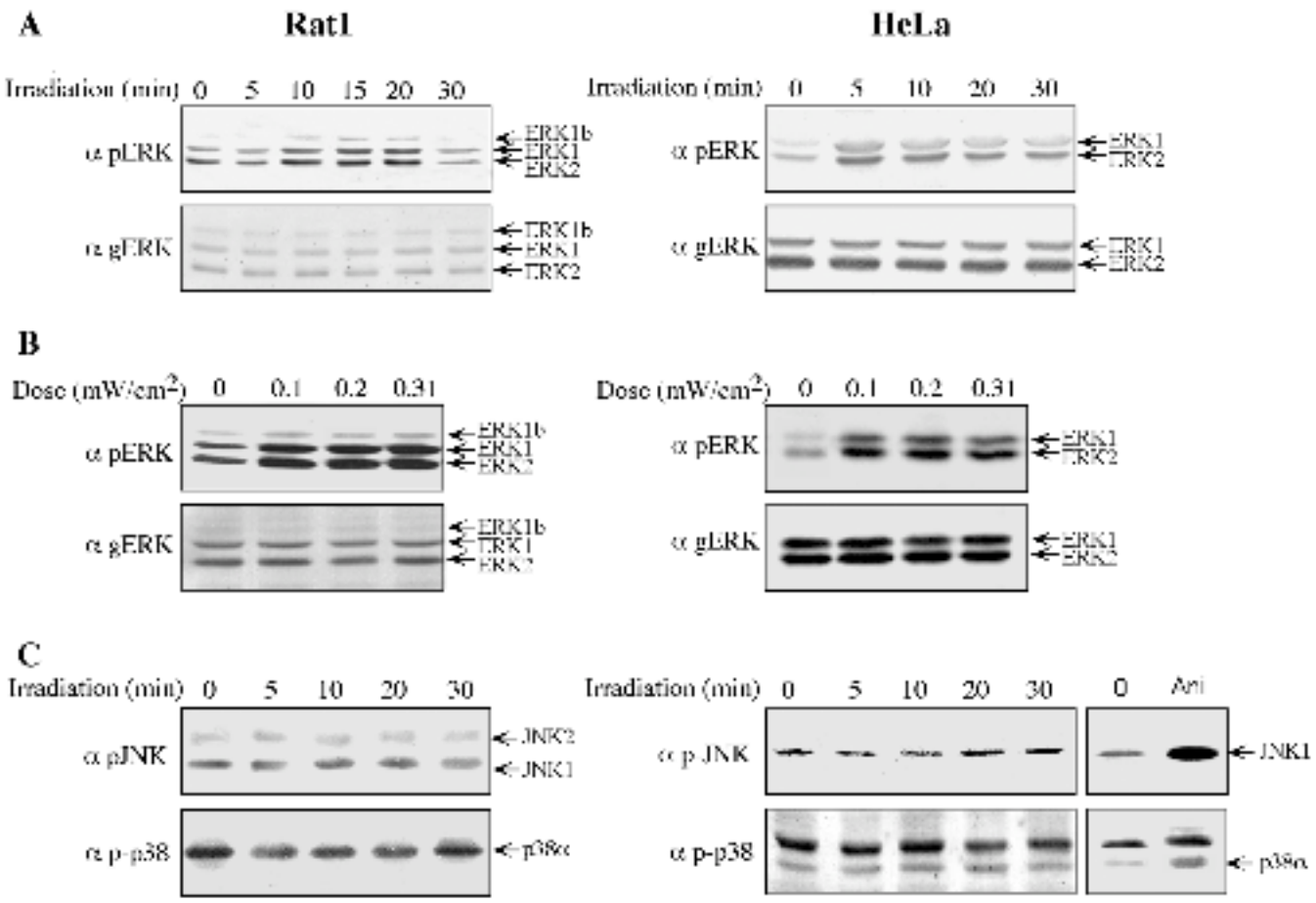


Fig. 1

Biochemical Journal Immediate Publication. Published on 25 Apr 2007 as manuscript BJ20061653

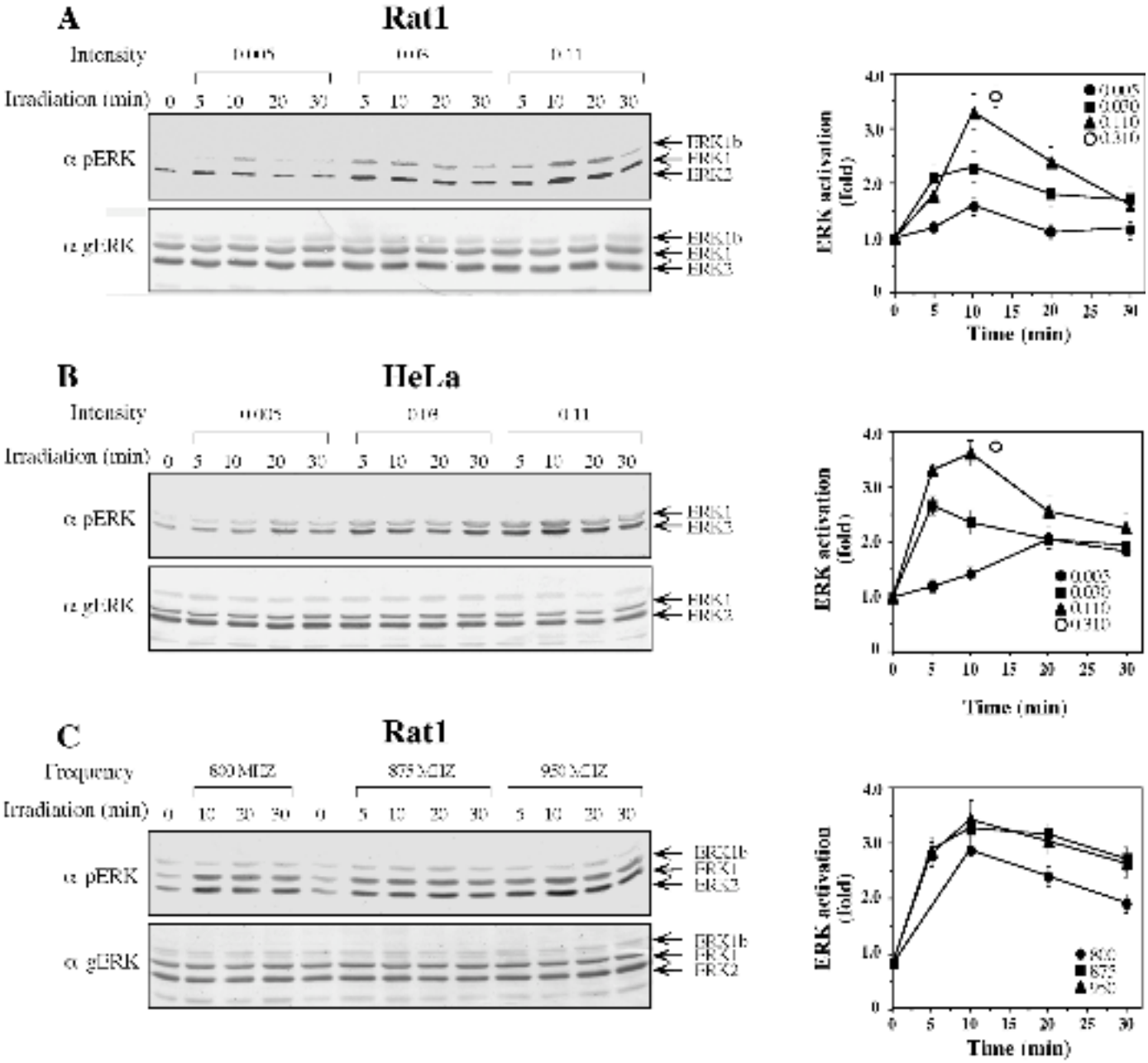


fig. 2

Biochemical Journal Immediate Publication. Published on 25 Apr 2007 as manuscript BJ20061653

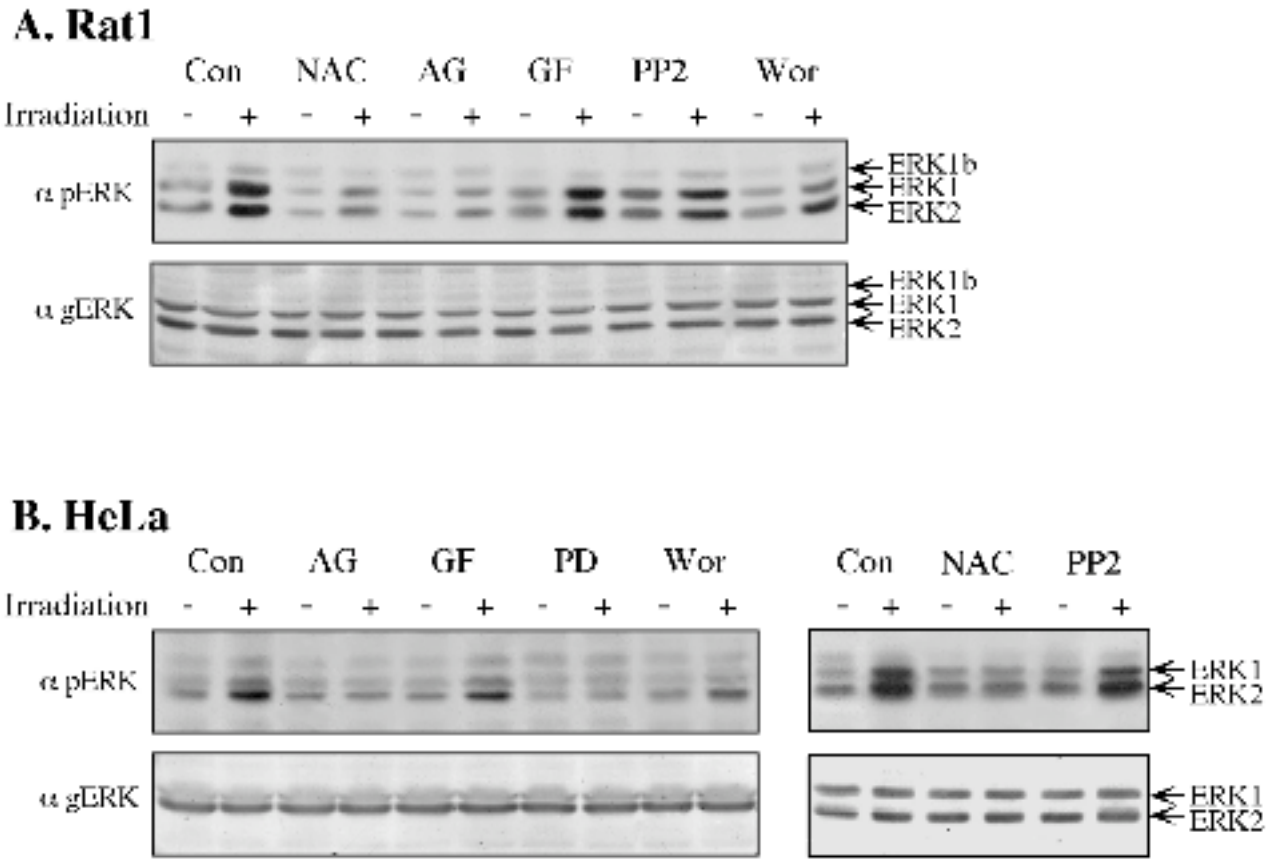


fig. 3

Biochemical Journal Immediate Publication. Published on 25 Apr 2007 as manuscript BJ20061653

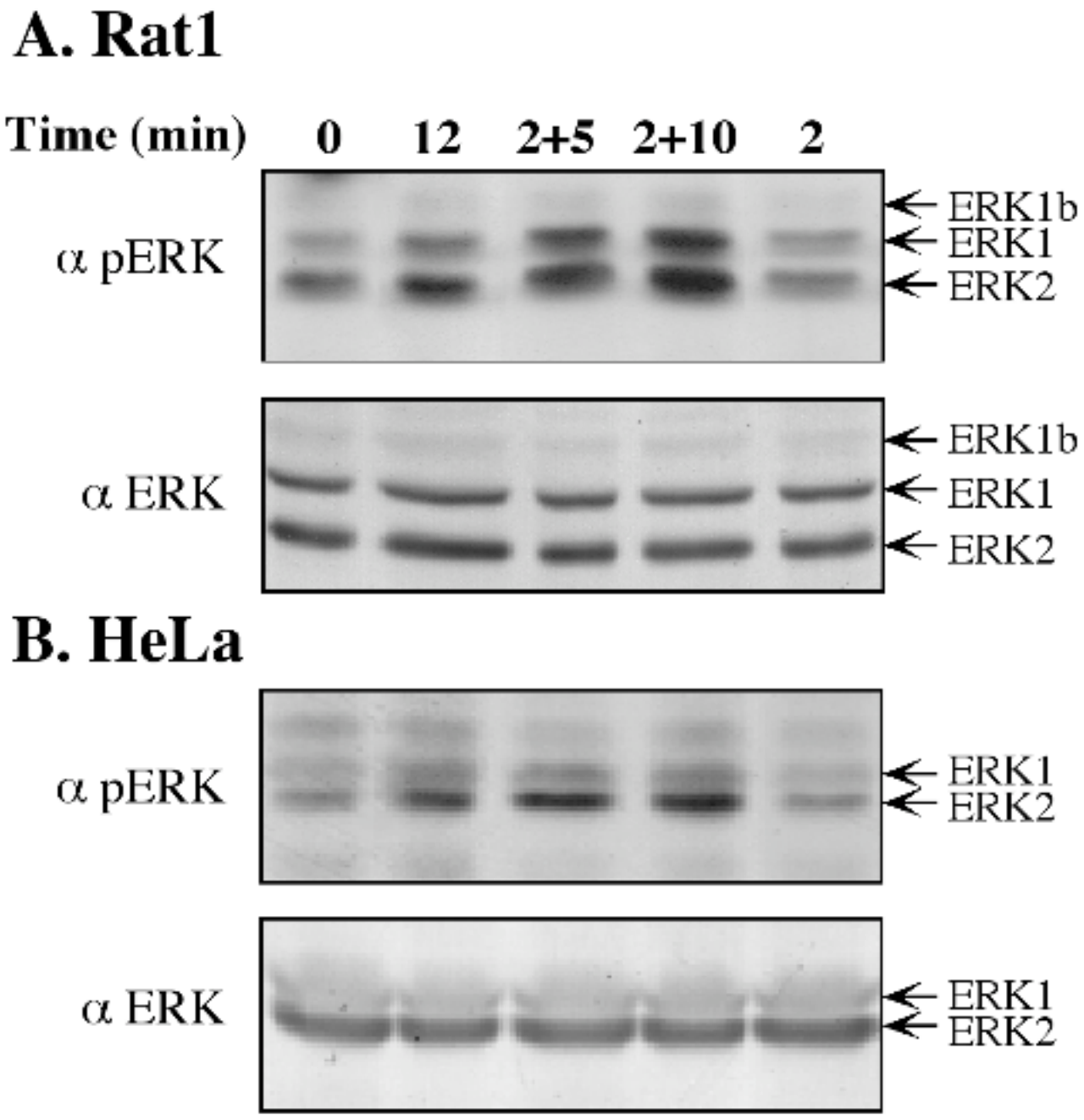


fig. 4

Biochemical Journal Immediate Publication. Published on 25 Apr 2007 as manuscript BJ20061653

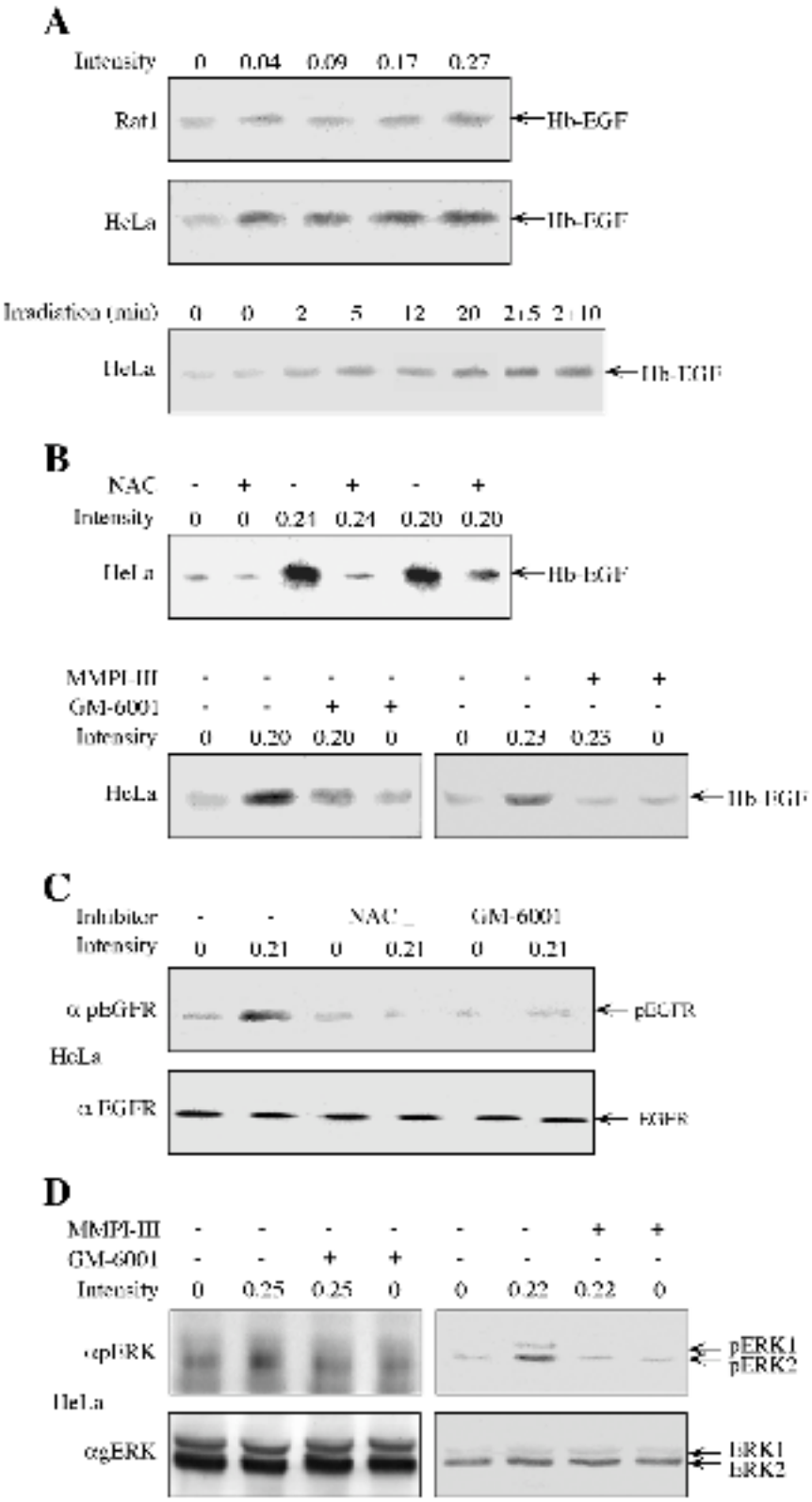


Fig. 5

Biochemical Journal Immediate Publication. Published on 25 Apr 2007 as manuscript BJ20061653

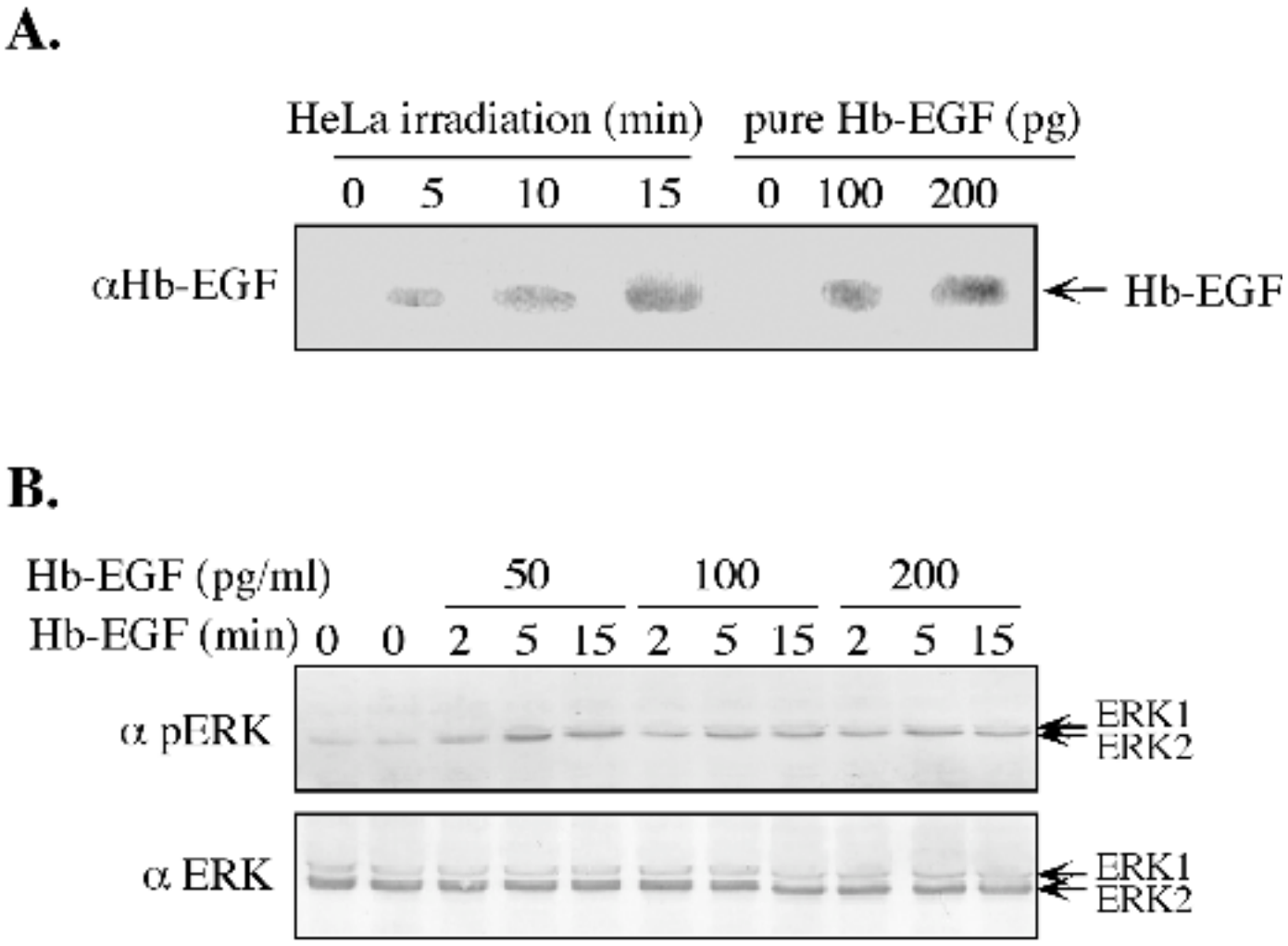
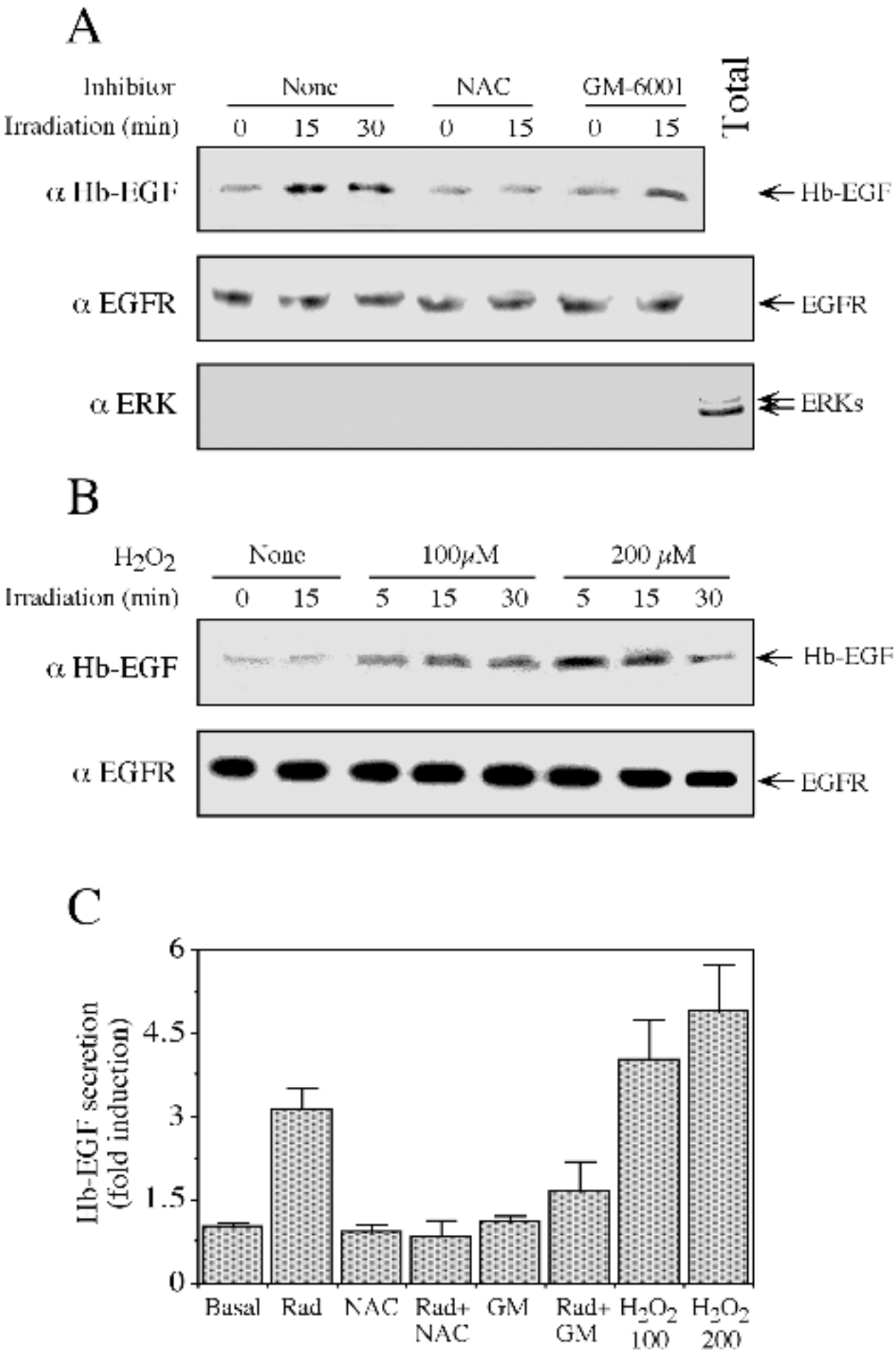


Fig. 6

Biochemical Journal Immediate Publication. Published on 25 Apr 2007 as manuscript BJ20061653

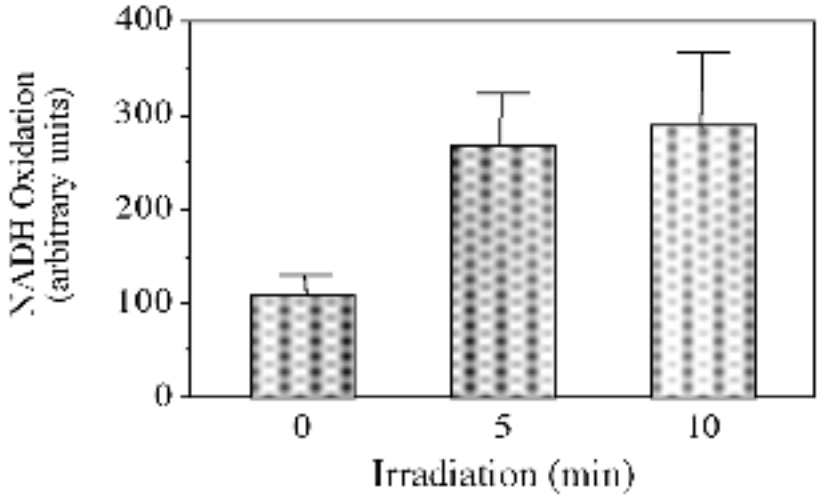
Fig. 7



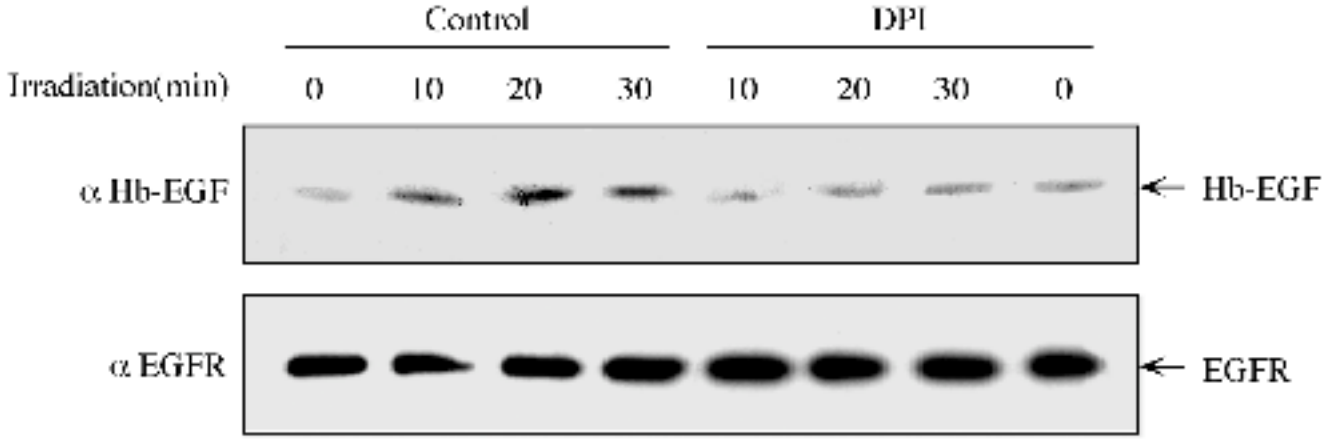
Biochemical Journal Immediate Publication. Published on 25 Apr 2007 as manuscript BJ20061653

Fig. 8

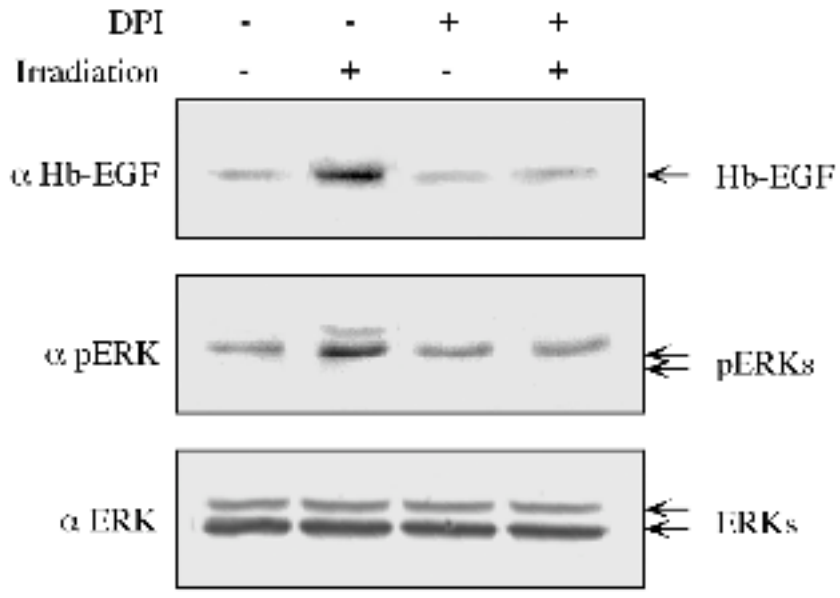
A. Membranes



B. Membranes



C. Cells



Biochemical Journal Immediate Publication. Published on 25 Apr 2007 as manuscript BJ20061653

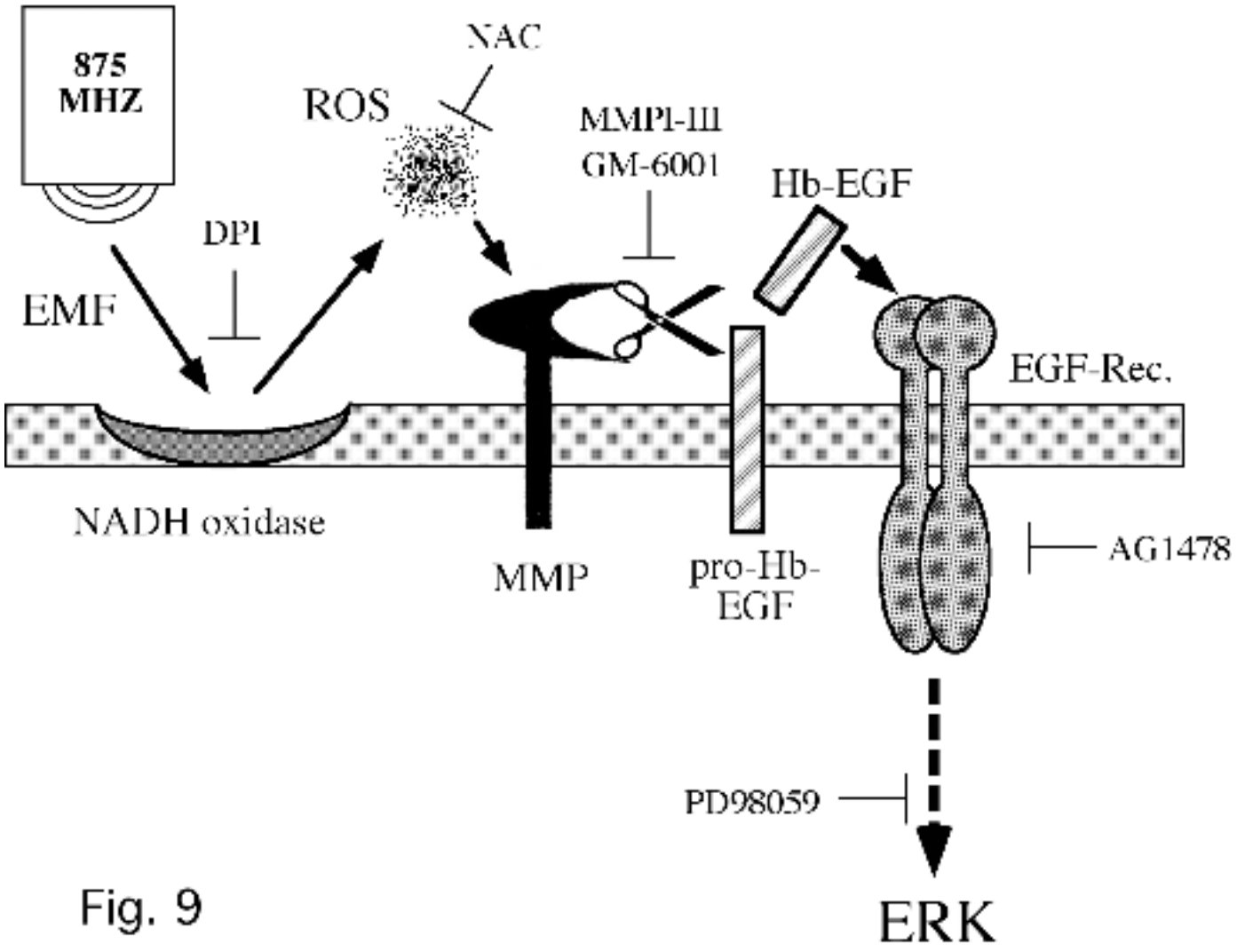


Fig. 9