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

Joseph FRIEDMAN*, Sarah KRAUS*, Yirmi HAUPTMAN†, Yoni SCHIFF† and Rony SEGER*[1]

*Department of Biological Regulation, The Weizmann Institute of Science, Rehovot 76100, Israel, and †Gal Safe Ltd, Rishon Lezion, Israel 75153

The exposure to non-thermal microwave electromagnetic fields generated by mobile phones affects the expression of many proteins. This effect on transcription and protein stability can be mediated by the MAPK (mitogen-activated protein kinase) cascades, which serve as central signalling pathways and govern essentially all stimulated cellular processes. Indeed, long-term exposure of cells to mobile phone irradiation results in the activation of p38 as well as the ERK (extracellular-signal-regulated kinase) MAPKs. In the present study, we have 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 signalling 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

ROS (reactive oxygen species). These ROS then directly stimulate MMPs (matrix metalloproteinases) and allow them to cleave and release Hb-EGF [heparin-binding EGF (epidermal growth factor)]. This secreted factor activates the 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 from mobile phones induces the activation of the ERK cascade and thereby induces transcription and other cellular processes.

Key words: extracellular-signal-regulated kinase (ERK), heparin-binding epidermal growth factor (Hb-EGF), matrix metalloproteinase (MMP), mobile phone irradiation, NADH oxidase, reactive oxygen species (ROS).

INTRODUCTION

The intensive use of mobile or cellular phones in the last 20 years has aroused concern about possible health problems that may be caused by the microwave irradiation which is associated with these phones [1]. The current safety standard for the use of mobile phones takes into consideration mainly heating, which is induced by their electromagnetic field. According to these parameters, which are measured by SARs (specific absorption rates; [2]), the amount of energy delivered is too low to trigger biological effects; however, it has been shown that electromagnetic fields can affect living tissues by energies that are much lower than those causing changes in the temperature of tissues [3]. These temperature-insensitive responses can influence the physiology of cells either in culture [4] or 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]. Similarly to the above systems, the higher-frequency (approx. 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, including c-Jun and c-Fos [10,12], and HSPs (heat-shock proteins) [13], such as HSP27 [3] and HSP70 [14,15], but not HSP90 levels that are

actually reduced [15]. The elevated expression of these proteins may participate in the induction of various cellular processes that appear to be affected by mobile phones [13], which include replication [4], cell-cycle progression [16] and apoptosis [15,17].

A major mechanism that regulates transcriptional activity in response to extracellular stimuli is the activation of the MAPK (mitogen-activated protein kinase) 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 the stress response [18–20]. Four canonical MAPK cascades have been identified so far. These are ERK (extracellular-signal-regulated kinase) 1/2, JNK (c-Jun N-terminal kinase) 1–3 [SAPK1 (stress-activated protein kinase 1)], p38MAPK (SAPK2) and BMK1 (big MAPK1; also known as ERK5). Each of the cascades is composed of three to six 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 has been shown that lengthy exposure to mobile phone irradiation can activate the p38MAPK, JNK and ERK cascades [3,21], although reduction in p38MAPK levels has also been reported [15]. These changes in activity of the MAPKs 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 frequencies. However, in most systems,

---

Biochemical Journal

the ERK cascade is known to be functional within a few minutes of activation [20], a time frame that has not been addressed for mobile phone irradiation and is the subject of the present study.

Using Rat1 and HeLa cells, as well as isolated membranes from 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 ROS (reactive oxygen species) that are produced upon irradiation by membranal NADH oxidase. The generated ROS then directly activates MMPs (matrix metalloproteinases) to release Hb-EGF [heparin-binding EGF (epidermal growth factor)], which further activates the ERK cascade. Thus the present study delineates, for the first time, a detailed molecular mechanism by which electromagnetic radiation at mobile phone frequencies induces short-term MAPK activation.

EXPERIMENTAL

Stimulants, inhibitors, antibodies and miscellaneous reagents

GF109203X, PD098059, SB203580, AG1478, PP2, anisomycin, MMPI-III, wortmannin and recombinant Hb-EGF were from Calbiochem; GM-6001 was from Biomol Research Laboratories; NAC (N-acetylcysteine) and DPI (diphenyleneiodonium), as well as heparin immobilized on agarose, were from Sigma. Mouse monoclonal anti-active MAPKs [phospho-ERK (pERK), phospho-JNK (pJNK) and phospho-p38MAPK (p-p38)], rabbit polyclonal anti-ERK and anti-JNK antibodies were from Sigma; and anti-EGFR (EGF receptor), anti-[phospho-EGFR(Tyr^{1173})] (pEGFR) and anti-Hb-EGF (C-18) antibodies were from Santa Cruz Biotechnology.

Cell culture, stimulation, harvesting and Western blotting

Rat1 and HeLa cells were obtained from the American Type Culture Collection and were grown in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10 % (v/v) heat-inactivated FCS (fetal calf serum; Gibco Laboratories), 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 °C. For experiments, the cells were serum-starved for 16 h prior to stimulation in medium containing 0.1 % FCS. Following treatment, the cells were washed twice with ice-cold PBS 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 the determination of the phosphorylation of MAPKs, cells were subsequently harvested in ice-cold homogenization buffer (buffer H; buffer A containing 1 mM benzamidine, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 2 μg/ml pepstatin-A), sonicated (50 W; 2×7 s), and the cell lysates were subjected to centrifugation (20 000 g for 15 min at 4 °C). The supernatant was assayed for protein content using the Coomassie protein assay (Pierce), and equal amounts of proteins were subjected to SDS/PAGE and Western blotting. The detection was carried out using either alkaline phosphatase (Promega) or ECL® (Amersham Biosciences) kits, according to the manufacturer's instructions.

Irradiation of cells

Subconfluent Rat1 of HeLa cells in 6-cm-diameter dishes or suspended membranes were irradiated inside the humidified incubator. A frequency generator (TGR1040 signal generator; Thurlby Thandar Instruments) and an amplifier of 1 W maximum (ERA-

3SM; Minicircuit) were used. The generator, located outside the incubator, was set to the desired power and connected to the power amplifier, which was connected to a panel antenna that was fixed in the incubator. The emitting antenna was placed in the centre of a shelf in the incubator at a distance of 10 cm from each plate. The walls of the incubator were covered with material absorbing irradiation to avoid reflection from the walls, and this created a homogeneous irradiating field. A field meter was used to measure the density of irradiation in mW/cm^2 in a particular area of the incubator. The temperature of the cell culture medium was measured throughout the experiment and was found to be unchanged ($\Delta < 0.05$ °C) even with the highest intensities used throughout the experiments.

After irradiation, the cells were washed, harvested and subjected to Western blotting, as described above. To detect Hb-EGF, the medium of the cells was collected, Hb-EGF was enriched as described 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 performed using heparin–agarose beads. Briefly, 80 μl of beads [50 % (v/v) slurry] was added to 800 μl of the collected medium and incubated for 2 h at 4 °C under constant shaking. The beads were then washed sequentially, once with RIPA buffer [20 mM Tris/HCl (pH 7.4), 137 mM NaCl, 10 % (v/v) glycerol, 1 % Triton X-100, 0.5 % sodium deoxycholate, 0.1 % SDS, 2 mM EDTA, 1 mM PMSF and 20 μM leupeptin], twice with LiCl (0.5 M) and twice with buffer A. After the last wash, the beads were boiled in SDS/PAGE sample buffer (40 μl; 7 min), centrifuged (15 000 g for 1 min at 23 °C), and the supernatant containing Hb-EGF was subjected to Western blot analysis with the anti-(Hb-EGF) antibody.

Release of Hb-EGF from plasma membranes

HeLa cells were grown in 10-cm-diameter dishes to subconfluency, starved for 16 h in medium containing 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 at 3000 g for 10 min to remove the nuclei. The supernatant was centrifuged again at 10 000 g for 10 min to remove the mitochondria and other organelles, and then once at high speed (100 000 g for 30 min; Optima ultracentrifuge). The pellet containing purified plasma membranes was suspended in 1 ml of PBS to form a suspension. For each point in the irradiation experiment, 100 μl of the suspension was placed in a thin tube and irradiated. After irradiation, the samples were centrifuged again (100 000 g for 30 min) to remove the membranes, and the supernatant containing Hb-EGF was subjected to Western blotting, as above.

Activity of NADH oxidases in plasma membranes

Plasma membranes of HeLa cells were prepared as described above and suspended in 0.6 ml of reaction buffer containing 250 μM NADH in PBS. The suspended membranes (membranes of 6×10^6 cells for each reaction) were then either irradiated at 875 MHz at 37 °C for various times or left untreated, after which the samples were transferred to 4 °C. To determine NADH oxidase activity [22], the samples were incubated at 37 °C for 15 min, and changes of NADH absorption at 340 nM were detected using an Ultraspec 2000 spectrometer (Pharmacia Biotech).
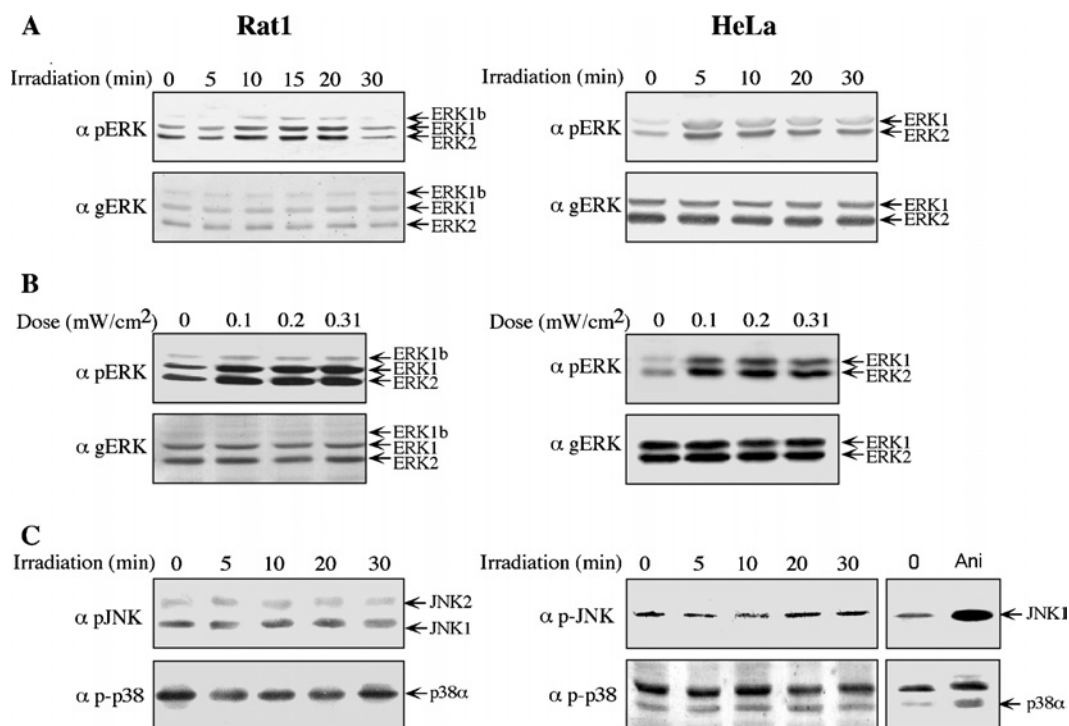
Figure 1   Mobile phone irradiation induces the phosphorylation of ERKs, but not that of JNKs or p38MAPKs

(**A**) Serum-starved Rat1 and HeLa cells were irradiated with a frequency generator at 875 MHz with an 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-pERK (α pERK) and anti-total ERK (α gERK) antibodies. (**B**) Serum-starved Rat1 and HeLa cells were irradiated at 875 MHz with intensities of 0.1, 0.2 and 0.31 mW/cm^2 for 10 min. Phosphorylation of ERKs was monitored as above. (**C**) Serum-starved Rat1 and HeLa cells were irradiated at 875 MHz with an intensity of 0.230 mW/cm^2 for the indicated times. As a positive control, serum-starved HeLa cells were also treated with 1 μg/ml anisomycin (Ani) for 15 min, after which the cells were washed and harvested as above. Phosphorylation of JNKs and p38MAPKs was detected with the indicated antibodies (α pJNK and α p-p38).

RESULTS

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 mobile phone irradiation. First, Rat1 and HeLa cells were subjected to mobile phone irradiation at a 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 approx. 0.45 mW/cm^2 in Israel. Under these conditions, the phosphorylation of ERKs was significantly increased in both Rat1 and HeLa cells (Figure 1). In Rat1 cells, phosphorylation peaked at 15 min after irradiation and returned to basal level within 30 min, whereas, in HeLa cells, peak phosphorylation was at 5 min after stimulation and decreased thereafter. Using the same frequency of 875 MHz, we then varied the intensity of irradiation and found that substantial phosphorylation of ERKs was already apparent at 0.10 mW/cm^2, and this was not elevated much more at higher intensities. As expected from many other stimuli [20], the total expression of ERKs did not change throughout the experiments. In contrast with the significant increase in the phosphorylation of ERKs, 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 (Figure 1C), the phosphorylation of p38MAPKα was even slightly reduced (Figure 1C) and ERK5 phosphorylation was not changed (results not shown). In HeLa cells, irradiation slightly decreased the phosphorylation

of JNK1 (Figure 1C) and had no effect on the phosphorylation of p38MAPKα (Figure 1C) and ERK5 (results not shown). The lack of effect of irradiation on the two stress-activated cascades, JNK and p38MAPK, indicates that the effect on ERKs is not stress-related and strengthens the notion that the temperature of the system was not changed upon irradiation.

Kinetics of the phosphorylation of ERKs

In view of the fact that the maximal phosphorylation of ERKs was already detected at a relatively low irradiation intensity of 0.10 mW/cm^2, we investigated the intensity and time–response effects further. Thus Rat1 and HeLa cells were irradiated at a frequency of 875 MHz with intensities of 0.005, 0.03 and 0.11 mW/cm^2 (Figure 2). A small increase in phosphorylation (1.4-fold in Rat1 cells and 2-fold in HeLa cells) was already detected at the lowest intensity; the phosphorylation was higher at 0.03 mW/cm^2 (2.2-fold in Rat1 cells and 2.7-fold in HeLa cells) and, at 0.11 mW/cm^2, was very close to the maximal phosphorylation induced by 0.31 mW/cm^2 (3.3-fold in Rat1 cells and 3.6-fold in HeLa cells). In addition to varying the irradiation intensity, we altered the frequency of irradiation in order to cover a wider range of irradiation used by mobile phones. Only small differences in the phosphorylation of ERKs were detected in Rat1 cells irradiated with an intensity of 0.07 mW/cm^2 of 800, 875 and 950 MHz. Interestingly, greater differences in phosphorylation in Rat1 cells were detected for the 46 kDa ERK1b [23], which appeared to be greatest at 875 MHz. Thus it is possible that ERK1b is more sensitive to differences in the frequency of irradiation than ERK1 and ERK2.
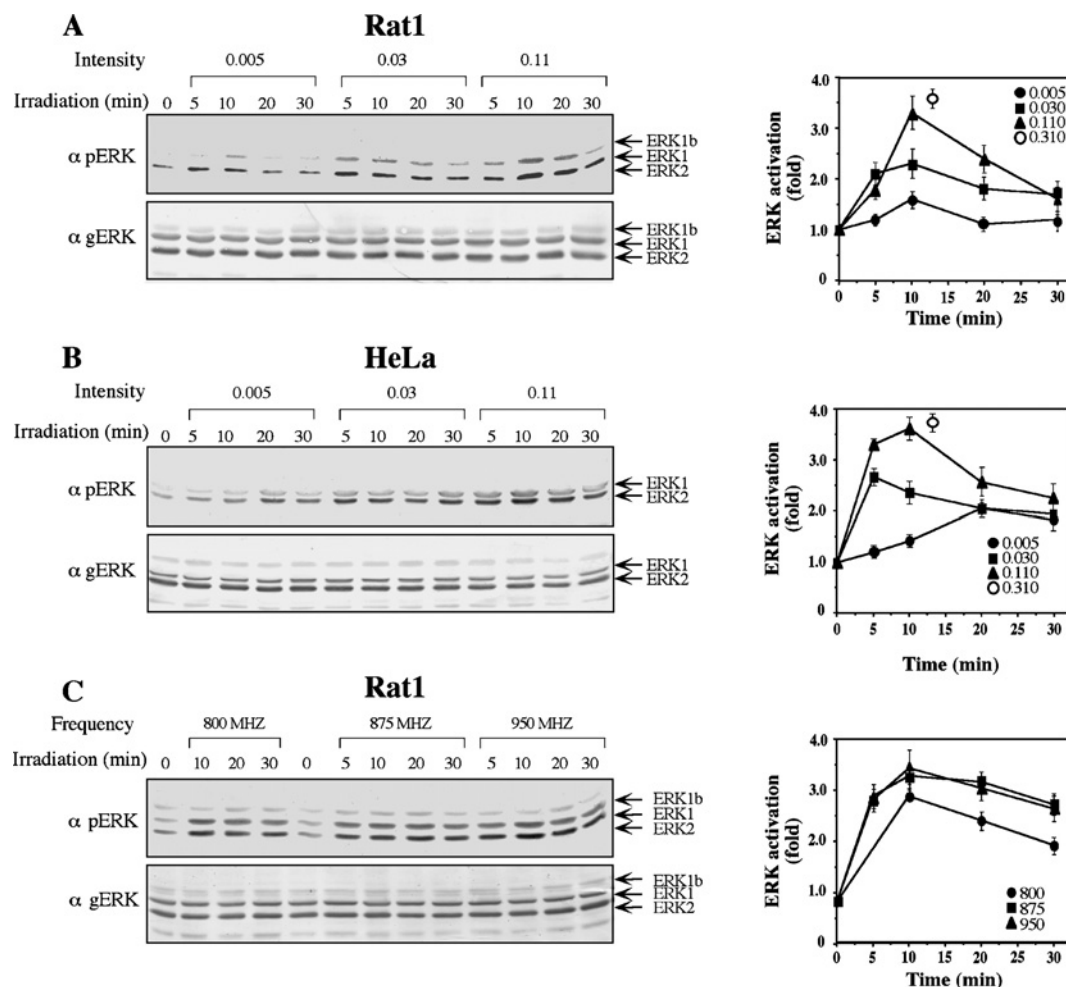
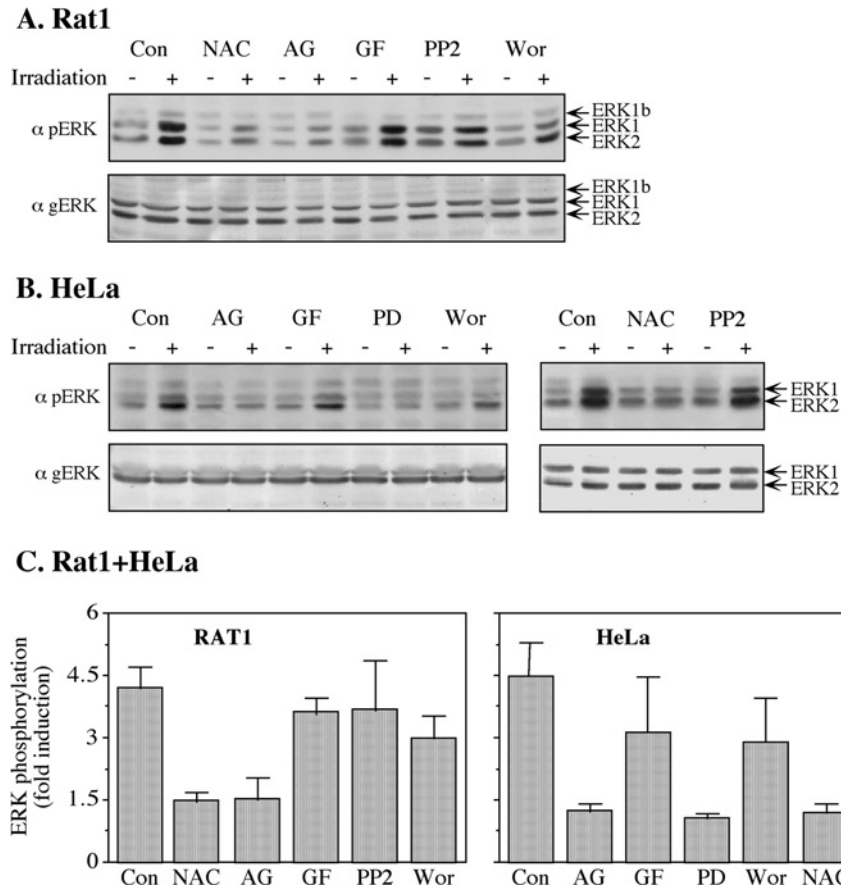**Figure 2   Kinetics of the phosphorylation of ERKs upon mobile phone irradiation**

(**A**) Serum-starved Rat1 cells were irradiated with a frequency generator at 875 MHz with intensities of 0.005, 0.03, 0.110 and 0.310 mW/cm² for the indicated times. The phosphorylation of ERKs was analysed as described in the legend to Figure 1, and shown for 0.005, 0.030 and 0.110 mW/cm². The results were quantified by densitometry, and the means ± S.E.M. of three experiments are shown in the right-hand panel. (**B**) Serum-starved HeLa cells were irradiated with at 875 MHz at intensities of 0.005, 0.03, 0.11 and 0.310 mW/cm². The phosphorylation of ERKs was followed as described in (**A**). (**C**) Serum-starved Rat1 cells were irradiated at 800, 875 and 950 MHz with an intensity of 0.070 mW/cm² for the indicated times. The phosphorylation of ERKs was followed as described in (**A**).

Irradiation-induced phosphorylation of ERK is mediated by ROS and EGFR

In order to study the possible mechanisms underlying the phosphorylation of ERKs in response to mobile phone irradiation, we used various signalling inhibitors, including NAC, which is a scavenger of ROS, the EGFR inhibitor AG1478, the PKC (protein kinase C) inhibitor GF109203X, the Src inhibitor PP2, the PI3K (phosphoinositide 3-kinase) inhibitor wortmannin and the MEK (MAPK/ERK kinase) inhibitor PD098059. Using 875 MHz irradiation at an intensity of 0.230 mW/cm² in both Rat1 and HeLa cells, we found that both NAC and AG1478 inhibited phosphorylation, whereas GF109203X and PP2 had no effect on the phosphorylation of ERKs in response to mobile phone irradiation (Figure 3). Wortmannin slightly inhibited the phosphorylation of ERKs in HeLa cells, but not in Rat1 cells, and, as expected, PD098059 inhibited the phosphorylation of ERKs in both HeLa (Figures 3B and 3C) and Rat1 (results not shown) cells. Thus all of these results clearly indicate that irradiation-induced phosphoryl-

ation of ERKs is mediated by EGFR and ROS, and PI3K may have a partial effect downstream of the receptor in HeLa cells.

Another indication for the mechanism of the phosphorylation of ERKs in response to mobile phone irradiation came from our finding that irradiation for 2 min was enough to exert the full effect on the phosphorylation of ERKs (Figure 4). Thus, when Rat1 or HeLa cells were irradiated at an intensity of 0.21 mW/cm² for different times, peak activity was detected 12 min after activation, but similar or even higher phosphorylation levels were obtained when the cells were irradiated for 2 min and then left in the incubator unirradiated for an additional 10 min. These results indicate that the initial event induced by irradiation is completed within 2 min and the signal that is induced by this event proceeds further to cause full ERK activation 10 min later. Alternatively, it is possible that the elevation in activity within 10 min after completing the irradiation is due to inhibition of phosphatases that normally counteract ERK activation [20]. Such inhibition of phosphatases could be due to the production of ROS, which are known to inhibit phosphatases of the MAPK cascades [24].

A. Rat1

B. HeLa

C. Rat1+HeLa

Figure 3   Use of inhibitors to identify mediators of irradiation-induced phosphorylation of ERKs

(**A**) Serum-starved Rat1 cells were incubated for 20 min with the following inhibitors: 2.5 mM NAC, 10 μM AG1478 (AG), 3 μM GF109203X (GF), 5 μM PP2, 250 nM wortmannin (Wor) or left untreated as a control (Con). After incubation, the cells were either irradiated with a frequency generator at 875 MHz with an intensity of 0.210 mW/cm^2 for 10 min (+) or left untreated (−). The phosphorylation of ERKs was detected as described in the legend to Figure 1. (**B**) Serum-starved HeLa cells were incubated for 20 min with the following inhibitors: 10 μM AG1478 (AG), 3 μM GF109203X (GF), 25 μM PD98059 (PD), 250 nM wortmannin (Wor), 2.5 mM NAC and 5 μM PP2 or left untreated as a control (Con). After incubation, the cells were either irradiated at 875 MHz with an intensity of 0.210 mW/cm^2 for 10 min (+) or left untreated (−). The phosphorylation of ERKs was detected as described in the legend to Figure 1. (**C**) Quantification of the inhibition experiments. Values are means ± S.E.M. for two or three experiments.

A. Rat1

B. HeLa
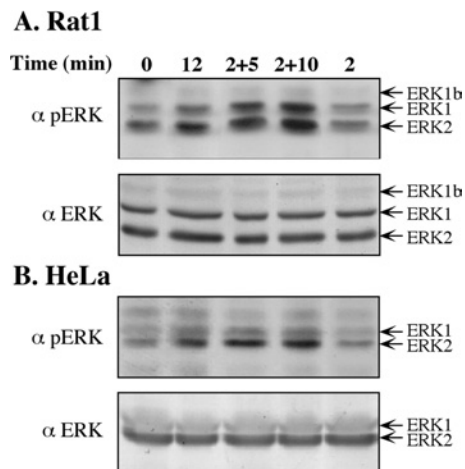
Figure 4   Continuous effect of irradiation on the phosphorylation of ERKs

Serum-starved Rat1 (**A**) or HeLa (**B**) cells were irradiated at 875 MHz with an intensity of 0.17 mW/cm^2 for 2 or 12 min, or were irradiated for 2 min and then left in the incubator without irradiation for an additional 5 or 10 min (2 + 5, 2 + 10). The phosphorylation of ERKs was detected as described in the legend to Figure 1.

## Role of MMPs and Hb-EGF in irradiation-induced phosphorylation of ERKs

In view of the finding that EGFR mediates the phosphorylation of ERKs in response to mobile phone irradiation, we attempted to elucidate the mechanism that mediates this process. One of the mechanisms studied was the cleavage and release of Hb-EGF by activated MMPs, which is a mechanism involved in the activation of ERKs by some GPCRs (G-protein-coupled receptors) and stress responses [25]. For this purpose, we irradiated Rat1 and HeLa cells with mobile phone irradiation at a frequency of 875 MHz for 10 min at various intensities and examined the release of Hb-EGF into the surrounding medium. Increases in Hb-EGF release upon mobile phone irradiation were detected in both Rat1 and HeLa cell lines (Figure 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 HeLa cells. The time course of Hb-EGF release in these cells was rapid, as a small elevation was already detected 2 min after irradiation. Consistent with ERKs activation, the release of Hb-EGF was increased further at later times and especially when the irradiation was for 2 min followed by further incubation without irradiation for 5 or 10 min (Figure 5A, lower panel). We then used specific signalling inhibitors to identify upstream

A

B

C

D
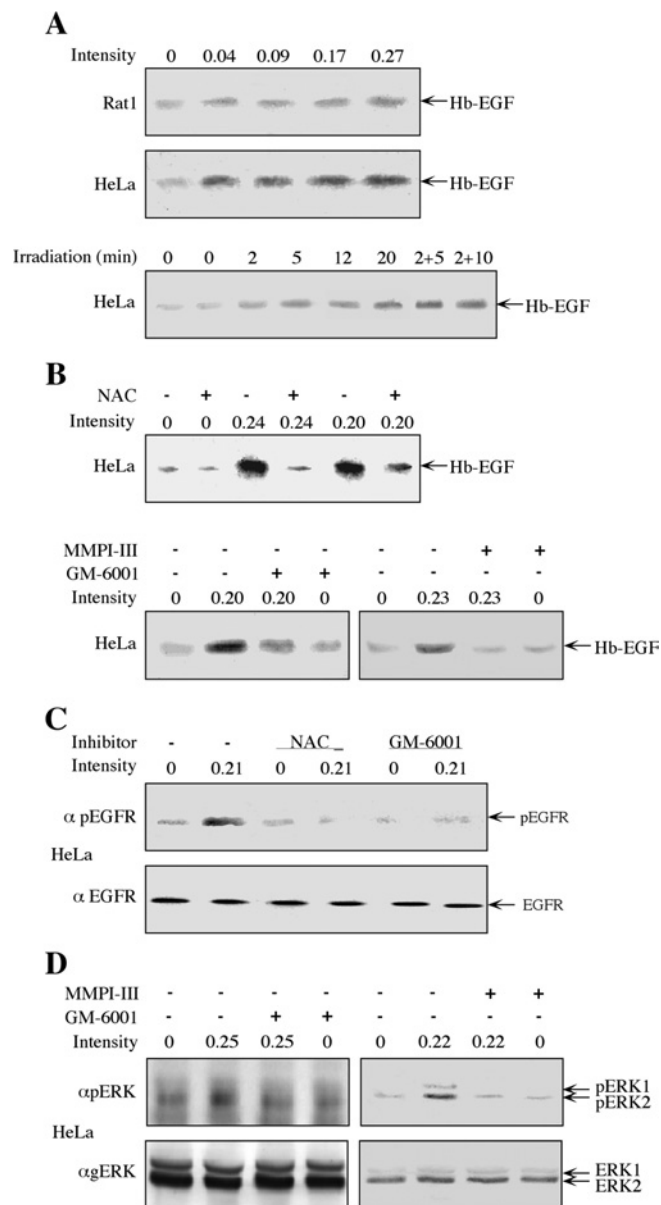
Figure 5   Involvement of Hb-EGF, MMPs and ROS in the irradiation-induced phosphorylation of ERKs

(**A**) Serum-starved Rat1 (top panel) and HeLa (middle panel) cells were irradiated at 875 MHz with an intensity of 0.04, 0.09, 0.17 and 0.27 mW/cm^2 for 10 min. For the time course determination, serum-starved HeLa cells were irradiated at 875 MHz with an intensity of 0.31 mW/cm^2 for the indicated times (bottom panel). After stimulation, the starvation medium was collected, Hb-EGF was enriched using heparin beads (as described in the Experimental section) and subjected to Western blot analysis with anti-Hb-EGF antibody. (**B**) Serum-starved HeLa cells were incubated for 20 min with 2.5 mM NAC (upper panel), 0.5 μM GM-6001 (lower panel, left) or 0.4 μM MMPI-III (lower panel, right), or left untreated as a control. The cells were then 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 were left untreated. One plate from each treatment was irradiated at 875 MHz with an intensity of 0.21 mW/cm^2 for 5 min, whereas the other plate was left untouched. The cells were harvested in RIPA buffer and subjected to Western blot analysis with anti-pEGFR (α pEGFR) or anti-EGFR (α EGFR) antibodies, as indicated. (**D**) Serum-starved HeLa cells were treated with GM-6001 (0.5 μM, 20 min), MMPI (0.4 μM, 20 min) or were left untreated. The cells were then irradiated at 875 MHz with an intensity of 0.25 or 0.22 mW/cm^2, as indicated. The phosphorylation of ERKs was detected as described in the legend to Figure 1.

components that led to this release. We found that the release of Hb-EGF from HeLa cells was blocked by NAC as well as by the MMP inhibitors GM-6001 and MMPI-III (Figure 5B), but not

A.

B.
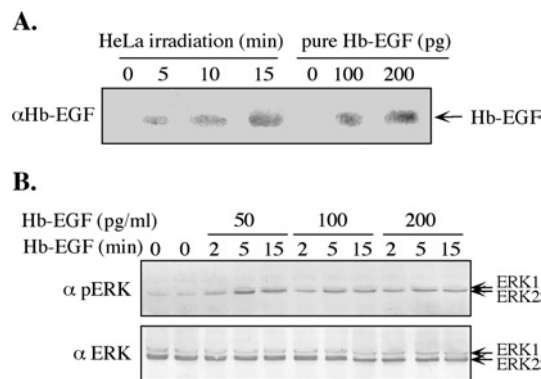
Figure 6   Amount of Hb-EGF released by irradiation is sufficient to induce the phosphorylation of ERKs
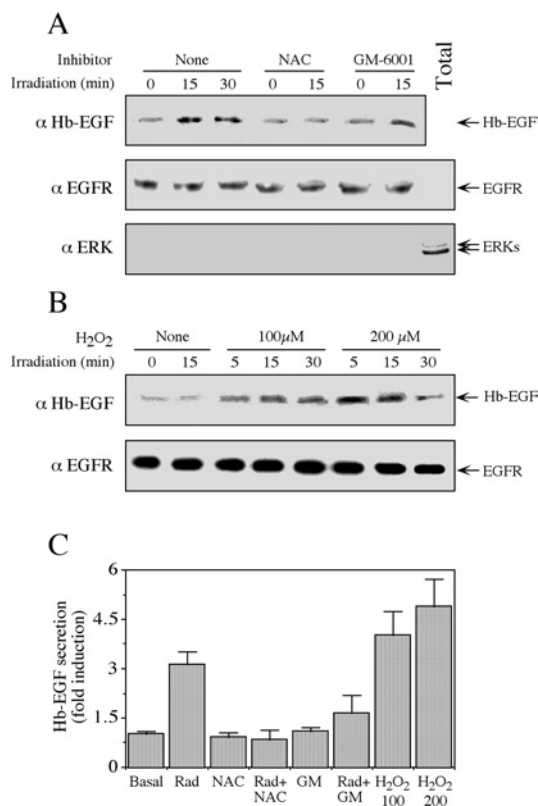
(**A**) Serum-starved HeLa cells were irradiated with 875 MHz at an intensity of 0.344 mW/cm^2 for 5, 10 and 15 min. Hb-EGF was enriched using heparin and subjected to Western blot analysis using anti-Hb-EGF antibodies (αHb-EGF), together with known concentrations of Hb-EGF (100 and 200 pg). (**B**) Low concentrations of Hb-EGF (50, 100 and 200 pg/ml) were used to stimulate ERKs in serum-starved HeLa cells. After stimulation for 2–15 min, the cells were harvested and subjected to Western blot analysis with anti-pERK (α pERK) and anti-total ERK (α ERK) antibodies.

by the EGFR inhibitor (results not shown). As expected, EGFR phosphorylation was induced by irradiation as well (Figure 5C), and, similarly to the Hb-EGF release, this phosphorylation was inhibited by NAC and GM-6001. Finally, NAC (Figure 3) and the MMP inhibitors GM-6001 and MMPI-III (Figure 5D) also inhibited the phosphorylation of ERKs upon irradiation of HeLa cells, supporting the involvement of MMPs upstream of EGFR/ERK activation.

To support further the involvement of Hb-EGF in ERK activation, we undertook to estimate the amount of released Hb-EGF and to verify the ability of such an amount to induce ERK activation. This was done by concentrating the Hb-EGF released into 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 (results not shown). Western blot analysis of the concentrated Hb-EGF, compared with known amounts of commercial, purified Hb-EGF, revealed that the concentration of released Hb-EGF at 5 min after irradiation was approx. 70 pg/ml, increasing to approx. 220 pg/ml at 15 min after irradiation (Figure 6). These concentrations could indeed induce significant phosphorylation of ERKs (Figure 6B), similar in magnitude to the phosphorylation induced by irradiation itself (Figure 1). Taken together, these data indicate that the phosphorylation of ERKs following mobile phone irradiation include production of ROS that activate MMPs and, consequently, the 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 been reported previously 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 studied whether the release of Hb-EGF in response to mobile phone irradiation can be carried out in purified plasma membranes without the presence of cytoplasmic components. To examine this, we purified plasma membranes from serum-starved HeLa cells and treated them with mobile phone irradiation at 875 MHz (0.300 mW/cm^2). We found
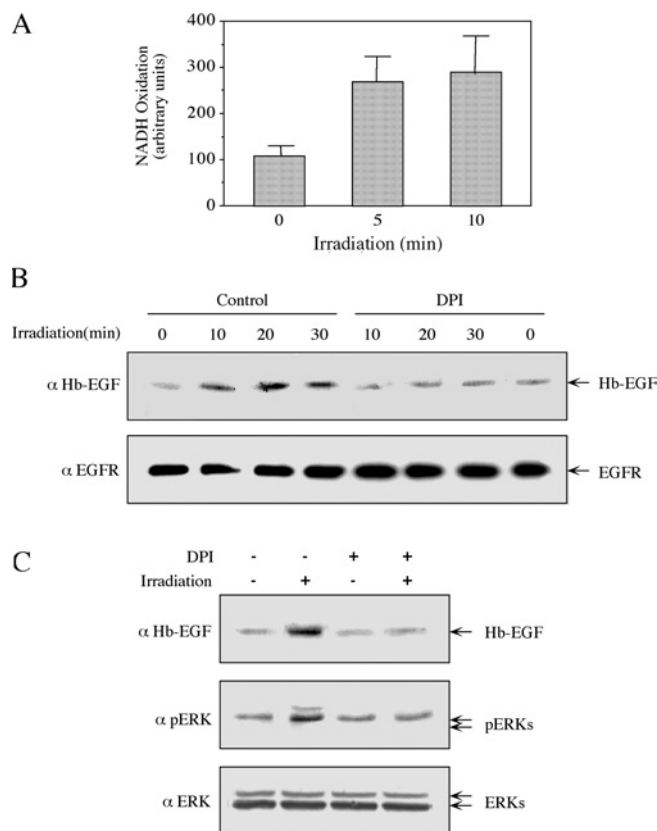
Figure 7   Irradiation induces the release of Hb-EGF from isolated plasma membranes

(**A**) Plasma membranes of serum-starved HeLa cells were isolated as described in the Experimental section. Membranes (5 μl of net membranes dissolved in 100 μl of PBS in each condition) were incubated with NAC (2.5 mM) or GM-6001 (0.5 μM), or were left untreated (None) for 15 min. Membranes were then irradiated at 875 MHz with an intensity of 0.300 mW/cm^2 for the indicated times and subjected to Western blot analysis with the anti-(Hb-EGF) antibody (α Hb-EGF; top panel). The membranes, as well as total cell extract (Total), were also analysed with the anti-EGFR (αEGFR) and -(total ERK) (αERK) antibodies (middle and bottom panels respectively). (**B**) Isolated plasma membranes from serum-starved HeLa cells, prepared as above, were incubated with 100 and 200 μM H$_2$O$_2$ for the indicated times. The amount of Hb-EGF and EGFR was determined with the appropriate antibodies. (**C**) Quantification of Hb-EGF secretion. Values are means $\pm$ S.E.M. for three separate experiments. GM, GM-6001; Rad, radiation.

that Hb-EGF was released from these membranes at 15 and 30 min after irradiation, and this release was significantly reduced when the membranes were pre-incubated with the inhibitors NAC and GM-6001 (Figure 7A, top panel). The purity of the membranes was confirmed by probing the isolated membranes with anti-ERK and anti-EGFR antibodies, which clearly showed that the membranes were devoid of any significant cytoplasmic impurities (Figure 7A, middle and bottom panels). These results indicate that ROS are activating MMPs at the plasma membrane, a process that does not appear to require the involvement of cytoplasmic components. A further validation of this hypothesis came when we incubated isolated membranes from serum-starved HeLa cells with H$_2$O$_2$. We found that this treatment also resulted in a release of Hb-EGF without significantly changing the amount of EGFR (Figure 7B). Quantification of these experiments (Figure 7C) emphasized the significant effects of irradiation, H$_2$O$_2$ and the inhibitors, confirming the sequential role of ROS and MMPs in the release of Hb-EGF.

Next, we investigated what the component in the plasma membrane was that was able to sense the mobile phone irradiation and convert it into ROS production. A membranal enzyme that can generate ROS is NADH oxidase, which can be activated by

Figure 8   NADH oxidase is involved in the irradiation-induced release of Hb-EGF and phosphorylation of ERKs

(**A**) Plasma membranes from serum-starved HeLa cells were isolated as described in the Experimental section. Membranes (5 μl of net membranes dissolved in 600 μl of buffer containing 250 μM NADH in PBS) were irradiated at 875 MHz with an intensity of 0.240 mW/cm^2 for the indicated times. NADH oxidase activity was determined as described in the Experimental section. Results are means $\pm$ S.E.M. for three independent experiments. (**B**) Plasma membranes of serum-starved HeLa cells were either incubated with 12 μm DPI (15 min) or left untreated, as indicated. The membranes were then irradiated at 875 MHz with an intensity of 0.200 mW/cm^2 for the indicated times. The amount of Hb-EGF and EGFR was analysed by Western blotting with the indicated antibodies. (**C**) Serum-starved HeLa cells were incubated with 12 μM DPI for 30 min or left untreated as a control. Cells were then irradiated at 875 MHz with an intensity of 0.200 mW/cm^2 for 10 min. The amount of Hb-EGF released and phosphorylation of ERKs was determined with the indicated antibodies, as described in the legends to Figures 1 and 5.

various other cellular processes [28,29]. To examine the possible role of this enzyme in our system, we examined whether membranal NADH oxidase was activated upon irradiation. Indeed, we found that irradiation of isolated membranes for 5 and 10 min significantly increased NADH oxidation activity (Figure 8A), indicating that, as reported previously [30], NADH oxidase does exist in purified HeLa membranes and we show in the present study that its activity is induced upon exposure to radiation. NADPH did not serve as a substrate in the isolated membranes (as reported in [30]), and the activity towards NADPH was not enhanced by irradiation (results not shown). To show that the membranal NADH oxidase can induce Hb-EGF release we used DPI, which is a selective inhibitor of NADPH oxidase [31]. Pretreatment of isolated membranes with DPI significantly decreased the release of Hb-EGF without affecting EGFR (Figure 8B). This inhibitory effect was also observed in intact HeLa cells, where DPI decreased the release of Hb-EGF and the phosphorylation of ERKs upon irradiation (Figure 8C). Therefore it is likely that irradiation in HeLa cells is sensed by a membranal NADH oxidase, which is activated to induce the rest of the ROS/ERK pathway.

DISCUSSION

In the present study, we have investigated the possible involvement of MAPK signalling in the cellular processes induced by irradiation from electromagnetic fields associated with mobile phones. Although activation of ERKs and p38MAPKs in response to mobile phone irradiation has been reported previously 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 was activated within 5 min, and the peak activity was within 10–15 min of irradiation (Figures 1 and 2). On the other hand, no phosphorylation of JNKs or p38MAPKs was detected in these short time intervals, although, similarly to the previous reports [3], longer exposures (4 h) induced a 2–3-fold activation of the stress-related p38MAPKs (results not shown). These results indicate that, prior to the stress response, mobile phone irradiation induces an immediate effect in the cytoplasm which activates ERK signalling to induce further transcription of a variety of genes [20].

The rapid induction of the ERK cascade raises the question as to what the mechanism involved in the perception or sensing of mobile phone irradiation might be and how the signal is transmitted to the intracellular signalling machinery. Although mobile phones can induce thermal effects that are thought to activate cellular events [32], it is unlikely that this is the cause of the activation of ERKs in the present study. First, it is unlikely that the irradiation intensity of 0.005 W/cm^2 for 5 min, which is enough to induce the phosphorylation of ERKs, can change the temperature of the medium, and, indeed, no change in temperature could be detected in the medium, even with higher intensities used in our experiments. Secondly, the fact that the stress-related cascades, which are known to be activated by heat or other related stresses, were not activated in the time course of our experiments indicates that the activation of ERKs is induced by other mechanisms. This fact, together with the relatively long wavelength of mobile phone irradiation, necessitated the identification of the molecular mechanisms of irradiation-stimulated phosphorylation of ERKs, which is the subject of the present study.

Our results using signalling inhibitors and isolated plasma membranes suggest that the phosphorylation of ERKs is induced through several steps (Figure 9). The first step in the processes appears to be irradiation-induced generation of ROS, probably due to activation of plasma-membrane-associated NADH oxidase (Figure 8). The ROS generated then activate membranal MMPs, which, in turn, cleave the anchored pro-Hb-EGF and thereby allow shedding of Hb-EGF into the surrounding medium. Finally, the Hb-EGF released binds to EGFR and activates it to induce the phosphorylation of ERKs 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 in the present study to be induced by the activation of NADH oxidase, which appears to be an early acceptor of mobile phone irradiation. These events appear to occur within seconds and initiate the subsequent activation of the pathway that leads to the phosphorylation of ERKs.

Several components of the proposed model (Figure 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 Hb-EGF and its binding to EGFR [36]. Similarly to GPCRs, irradiation and ROS appear to activate intracellular signalling pathways via stimul-
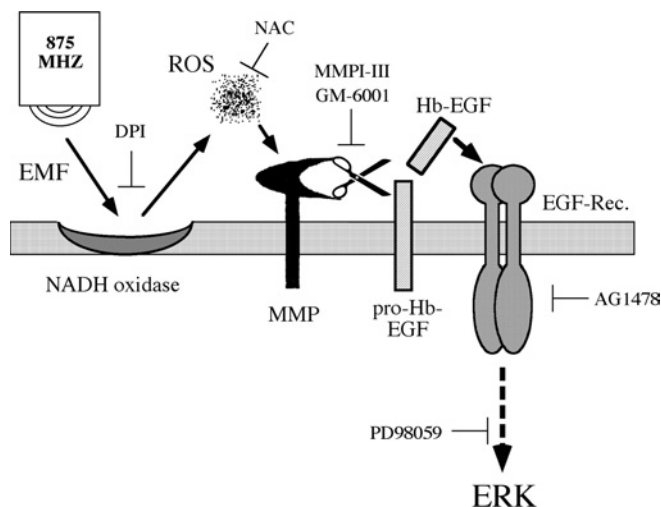
Figure 9 Schematic representation of the proposed mechanism that mediates the phosphorylation of ERKs upon mobile phone irradiation

This pathway is mediated by irradiation-induced activation of NADH oxidase which generates ROS at the plasma membrane. ROS then directly activate MMPs to cleave and release Hb-EGF, which binds to EGFR and activates the ERK cascade.

ation 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 last few years. These mechanisms included mainly activation of intracellular components, such as inhibition of protein tyrosine phosphatases [27] and induction of caveolar-induced cytoplasmic events [39]. Another suggested mechanism involves a covalent dimerization of 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 do not contain the cytosolic components (Figure 7) and no dimerization of EGFR was detected upon irradiation (results not shown). On the other hand, the inhibition of Hb-EGF production in plasma membranes by the ROS scavenger NAC and by MMP inhibitors (Figure 7) indicate that the effect of irradiation is mediated through activation of MMPs by generated ROS. This suggests a direct activation of MMPs by irradiation/ROS, which might act in a similar manner to UVB-irradiation-stimulated activation of MMP1 and MMP3 in human dermal fibroblasts [41].

One of the interesting questions raised in our present study is the identity of the molecular acceptor of mobile phone irradiation. Our results (Figure 8) indicate that one such early acceptor may be NADH oxidase, which appears to be localized in the plasma membrane [29] and converts the irradiation into ROS, which subsequently activate MMPs. This mechanism is different from the pathways suggested in other reports in which NADH oxidase expression is induced downstream of irradiation/ROS and possibly also downstream of ERKs themselves. For example, it has been 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, was induced in a p47^{phox}-containing NADH oxidase [43]. Finally, another example of such a mechanism is the involvement of EGFR transactivation in the up-regulation of NOX1, a catalytic subunit of NADH oxidase [44]. Therefore our findings are the first to demonstrate a role of membranal NADH oxidase in the early conversion of radiation energy into the production of ROS, which, in turn, initiate intracellular signalling cascades.

An important question that is raised from the activity of NADH oxidase in the isolated membranes that were relatively free of cytoplasmic components (Figure 7) is the identity of the electron donors to the system. Normally, NADPH oxidases use NADPH for this purpose, 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 suggestion 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 bilayer of the plasma membrane. Therefore the reduced quinones (hydroquinones) are the natural substrates for the NADH oxidase in HeLa cell plasma membranes and are likely to be used in the plasma membranes of the HeLa cells in our experiments. Furthermore, as it was shown that membranal NADH oxidase by itself can initiate intracellular signalling pathways [29], it is possible that this direct activation or the ROS generated 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 (Figure 3) is a result of an independent mechanism acting in parallel with the EGFR/Ras pathway.

In summary, we have shown in the present study 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 irradiation, and directly activate MMPs. In turn, the activated MMPs cleave and release Hb-EGF, which then binds to EGFR, activating it, thereby stimulating the ERK cascade. These studies demonstrate for the first time a detailed molecular mechanism for electromagnetic-irradiation-induced MAPK activation.

REFERENCES

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

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. et al. (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, III, L. H., 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. et al. (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, Jr, F. J. 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_2O_2 regulates the activation of distinct MAPK pathways. Free Radical 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 2006, 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. Science 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. et al. (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