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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

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Abstract We have examined whether non-thermal exposures of cultures of the human endothelial cell line EA.hy926 to 900 MHz GSM mobile phone microwave radiation could activate stress response. Results obtained demonstrate that 1-hour non-thermal exposure of EA.hy926 cells changes the phosphorylation status of numerous, yet largely unidentified, proteins. One of the affected proteins was identified as heat shock protein-27 (hsp27). Mobile phone exposure caused a transient increase in phosphorylation of hsp27, an effect which was prevented by SB203580, a specific inhibitor of p38 mitogen-activated protein kinase (p38MAPK). Also, mobile phone exposure caused transient changes in the protein expression levels of hsp27 and p38MAPK. All these changes were non-thermal effects because, as determined using temperature probes, irradiation did not alter the temperature of cell cultures, which remained throughout the irradiation period at 37 ± 0.3 °C. Changes in the overall pattern of protein phosphorylation suggest that mobile phone radiation activates a variety of cellular signal transduction pathways, among them the hsp27/p38MAPK stress response pathway. Based on the known functions of hsp27, we put forward the hypothesis that mobile phone radiation-induced activation of hsp27 may (i) facilitate the development of brain cancer by inhibiting the cytochrome c/caspase-3 apoptotic pathway and (ii) cause an increase in blood-brain barrier permeability through stabilization of endothelial cell stress fibers. We postulate that these events, when occurring repeatedly over a long period of time, might become a health hazard because of the possible accumulation of brain tissue damage. Furthermore, our hypothesis suggests that other brain damaging factors may co-participate in mobile phone radiation-induced effects.

Key words mobile phone · RF-EMF · non-thermal · phosphorylation · hsp27 · p38MAPK

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Introduction

The rapid increase in the use of mobile phones has brought about an urgent need to determine whether their emitted microwave radiation (radio-frequency-modulated electromagnetic fields; RF-EMF) could cause health hazards. The possibility of such effects remains controversial. However, if proven, it would necessarily lead to re-evaluation of the present RF-EMF radiation safety limits. Especially, the possibilities of induction of brain cancer, disturbances in functioning of blood-brain barrier, and the long-term exposure effects are considered by scientists and the general public to be of great importance (Jokela et al., 1999; The Royal Society of Canada Report, 1999; Stewart Report, 2000; Hyland, 2000; Zmirou Report, 2001).

The present mobile phone radiation safety standards are set so that the RF-EMF radiation absorbed by the living tissue does not cause heating and thus does not cause detrimental health effects (for review see Jokela et al., 1999; The Royal Society of Canada Report, 1999; Stewart Report, 2000; Zmirou Report, 2001). On the other hand, there appears to be sufficient evidence that RF-EMF, although not causing tissue heating, can cause non-thermal biological effects (for review see Jokela et al., 1999; The Royal Society of Canada Report, 1999;

Stewart Report, 2000; Zmirou Report, 2001). Whether these non-thermally-induced biological effects are strong enough to alter physiological processes is a matter of controversy. One of the causes of this uncertainty is that the possible biophysical mechanisms for RF-EMF interaction with living cells remain unknown. The energy deposited in tissue by a 900 MHz GSM mobile phone (4×10^{-6} eV) or by a 1800 MHz GSM mobile phone (7×10^{-6} eV) is far lower than the energy needed to break a chemical bond (1 eV) (Stewart Report, 2000). Therefore, some consider it questionable whether this low energy would be able to induce biological effects at all.

The early signs of cell response to a stress factor (e.g. chemical, radiation, heat, etc.) are changes in protein phosphorylation and activation of stress response (Ono and Han, 2000). There are several published studies examining effects of electromagnetic fields (60 Hz) on protein phosphorylation and activity of stress proteins and kinases (Pipkin et al., 1999; Jin et al., 2000; Morehouse and Owen, 2000; Woods et al., 2000; Wetzel et al., 2001). However, results of these studies are not directly applicable for the clarification of the possible biological and health effects of the RF-EMF radiation emitted by mobile phones. The effects of mobile phone radiation on cellular stress proteins were examined in only a few studies (Cleary et al., 1997; Fritze et al., 1997b; Daniells et al., 1998; de Pomerai et al., 2000; Kwee et al., 2001). Together, the results obtained in these studies suggest that, indeed, mobile phone radiation causes activation of cellular stress response and, possibly, related changes in cell physiology.

The aim of the present study was to determine whether non-thermal exposure of cells to 900 MHz GSM mobile phone radiation activates signal transduction pathways and induces cellular stress response in a human model. Using cultures of human endothelial cell line EA.hy926, we examined the overall change in protein phosphorylation, which is indicative for the activation of cellular signal transduction pathways. The effect on cellular stress response was examined by determining phosphorylation and expression status of one of the members of stress protein family – heat shock protein-27 (hsp27). Results obtained suggest that mobile phone radiation activates cellular signal transduction and stress response pathways, and therefore, it might be able to alter cell physiology and potentially cause a health hazard.

Methods

Cell culture

EA.hy926 cells (a gift from Dr. Cora-Jean S. Edgell, North Carolina University at Chapel Hill, NC, USA) (Edgell et al., 1983) were grown in Dulbecco's MEM, supplemented with antibiotics, 10% fetal bovine serum, L-glutamine, and HAT-supplement. For experiments, cells were removed from culture flasks with trypsine, washed, and seeded at a density of 1.2×10^6 cells per 55 mm-diameter glass petri dish (DURAN, Germany). After overnight culture, semi-confluent monolayers of EA.hy926 cells (Fig. 1A) were exposed to sham or RF-EMF radiation. Cell cultures for sham and irradiation were prepared in the same kind of glass dishes, derived from the same batch of cells, seeded at the same cell density, and grown for the same period of time before the experiment. The only difference between irradiated and sham samples was that the irradiated dishes resided for 1 hour in the incubation chamber with RF-EMF radiation turned on, whereas sham dishes resided in the irradiation chamber for the same period of time but with irradiation turned off.

RF-EMF exposure system and exposure protocol

Cells were irradiated with a simulated mobile phone microwave radiation in a specially constructed exposure system, based on the use of a high Q waveguide resonator operating in TE_{10} mode. The irradiation chamber (Fig. 1B) was placed vertically inside a cell-culture incubator with two 55 mm-diameter glass petri-dishes placed so that the E-field vector was parallel to the plane of the culture medium. Temperature-controlled water was circulated through a thin (9 mm) rectangular glass-fiber-molded waterbed underneath the petri dishes. In all experiments reported here, cells were exposed for 1 hour to a 900 MHz GSM signal at an average SAR of 2 W/kg. SAR values ranged from 1.8 W/kg to 2.5 W/kg depending on the area of the dish, caused by the non-uniform distribution of the RF-EMF radiation field. The average SAR level of 2 W/kg was selected because it is the safety limit for the mobile phone microwave radiation emission as defined by ICNIRP (International Commission on Non-Ionizing Radiation Protection). The RF-EMF signal was generated with an EDSG-1240 signal generator and modulated with a pulse duration of 0.577 ms and a repetition rate of 4.615 ms to match the GSM signal modulation scheme. The signal was amplified with RF Power Labs R720F amplifier and fed to the exposure waveguide via a monopole type feed post. The SAR distribution in the cell culture (Fig. 1C) and the E-field above the cell culture were determined using computer simulations (FDTD method). The simulations were done with a commercial XFDTD code (Remcom, USA) with simulation grid size of $3\times3\times3$ mm^3 in the main grid and $1\times1\times1$ mm^3 in the sub grid, consisting of the culture dishes and part of the waterbed. The maximum SAR was obtained in the center of the petri dish, decreasing to about 6 dB at the edges of dish. Simulation results were verified with measurements. The electric field in the air above the cell cultures was measured with a calibrated miniature Narda 8021B E-field probe. The measured E-field values differed less than 15% from the corresponding simulated E-fields. The SAR distribution was measured with small, calibrated temperature probes (Luxtron and Vitek) directly from the culture medium. The measurements were done at room temperature outside the incubator with increased culture medium height, in order to reduce the measurement uncertainty at the air-medium boundary. The temperature was measured (Vitek probe) for 10 sec in order to limit the effect of heat convection and conduction (Moros and Pickard, 1999). The Luxtron probe has a lower temperature resolution (0.1 °C) compared to the Vitek probe (0.001 °C), and thus, the temperature had to be measured for 1 min to achieve a sufficient rise in temperature (1 °C). Due to these short measurement times, the power fed to the chamber was increased up to 25 W, and the resulting SAR value was afterwards scaled down to 1 W of input power. The measured SAR values at the center of the culture medium (3-mm depth) were 2.5 W/kg (Luxtron) and 5.0 W/kg (Vitek). These values can be compared to the simulated value of 2 W/kg and 3.6 W/kg, respectively, with simulation parameters changed to correspond with the measurement situation. The measured values can be considered as the upper and lower limits of SAR, due to measurement uncertainties described above, and thus, they validate the simulations. The

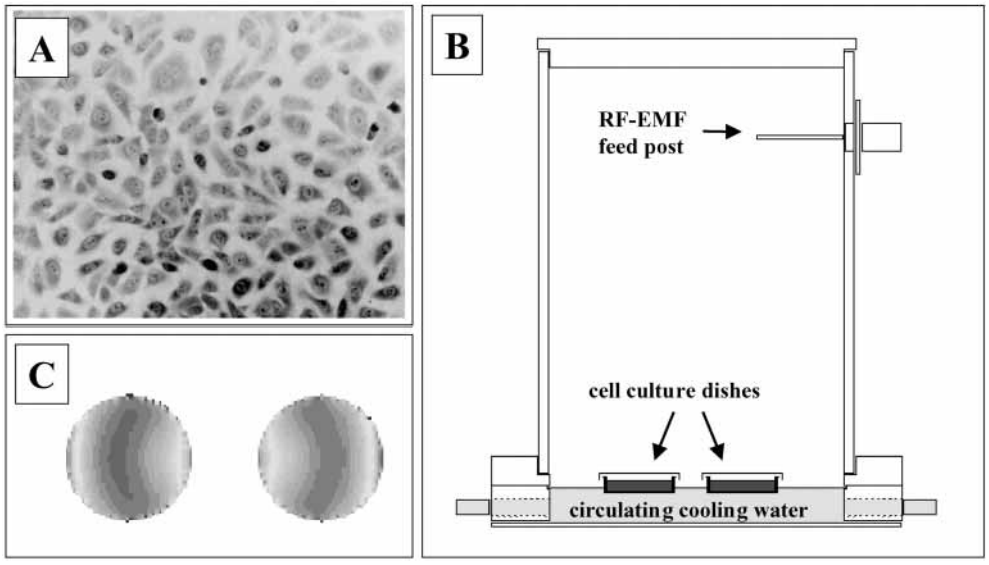

Fig. 1 (**A**) Phase-contrast image showing growth pattern and density of cell cultures on the day of sham or RF-EMF treatment. (**B**) Cross-section scheme of the RF-EMF exposure chamber. (**C**) Degree of uniformity of RF-EMF distribution in culture dishes simulated with XFDTD method. Culture medium height was 6 mm and maximum specific absorption rate (SAR) at the medium was 3.6 W/kg for 1 W input power. *Dark gray color* corresponds with the areas of highest SAR and *white* with the lowest SAR.

waveguide resonator's water-cooling system was tested by long-term temperature measurements using a Luxtron probe. The temperature was recorded twice a minute over the normal 1-hour exposure period at 2 W/kg. The temperature remained at 37 ± 0.3 °C during the whole measurement time. Therefore, the reported biological effects are of non-thermal nature.

Analysis of protein expression and phosphorylation

Immediately after the sham or RF-EMF exposure, culture dishes were placed on ice, washed with ice-cold phosphate-buffered saline (PBS) with 4 mM orthovanadate, and cells were detached by scraping and pelleted. Pellets were lysed in buffer consisting of 9.5 M urea, 2% CHAPS, 0.8% Pharmalyte pH 3–10 (Pharmacia, Sweden), 1% dithiothreitol, 1 mM phenylmethylsulphonyl-fluoride and 4 mM orthovanadate. Non-soluble cellular debris was removed from lysates by centrifugation for 1 hour at $42\,000 \times g$ at 15 °C. Protein content was determined with the Bradford method. Samples containing 125 µg of protein were loaded into the 11 cm IPG strips pH 3–10 by overnight re-swelling. The isoelectrofocusing (DryStrip Kit with Multiphor II apparatus; Pharmacia, Sweden) was carried out at 20 °C for 84 000 Volt-hours. SDS-PAGE of proteins in IPG strips was performed using a 20 cm long 8% gel in a ProteanIIxi Multicell apparatus (Bio-Rad, UK). Proteins were visualized in 2D-gels using standard Merril's silver-staining method with Morrissey's modification. Silver-stained gels were scanned while wet with a Bio-Rad GS-710 densitometer. In phosphorylation experiments, prior to RF-EMF or sham exposure, cell monolayers were washed with pre-warmed phosphate-free DMEM, supplemented with 10% dialyzed FBS, antibiotics, L-glutamine, and HAT. Just before the exposure, a 1 mCi/ml (5 ml/dish) of ^{32}P-orthophosphate (NEN, Belgium) was added to the cells. To detect the ^{32}P-phosphoproteins, the gels were dried and X-ray films were exposed at − 70 °C for up to 3 days. The computerized analyses of the protein distribution pattern in silver-stained 2D-gels and the distribution pattern of ^{32}P-phosphoproteins in 2D-autoradiograms were performed using PDQuest 6.1.0 software (Bio-Rad, UK).

Detection of hsp27 expression in cell cultures

Hsp27 was detected using the standard indirect immunofluorescence method. Cells were fixed in 3.7% buffered paraformaldehyde on ice for 10 min, and their membranes permeabilized with 0.5% Triton X-100 in PBS for 10 min at room temp. Non-specific binding sites were blocked with 5% bovine serum albumin (BSA) in PBS (PBS/BSA) for 30 min. To visualize, hsp27 cells were incubated for 1 h with anti-hsp27 antibody (ImmunoCruz, CA, USA), diluted 1:200 with PBS/BSA, then washed, and incubated for 30 min with FITC-conjugated goat anti-mouse Ig (DAKO, Denmark), diluted 1:50 with PBS/BSA, then washed. Specimens were observed using a Leitz fluorescence microscope, ERGOLUX AMC. Images were acquired using a digital camera IMAC-CCD S30 and Metasystems ISIS In Situ Imaging System (ISIS, Germany).

Detection of hsp27 phosphorylation

To determine changes in protein phosphorylation, ^{32}P-ortophosphate was present in the cultures during the 1-hour sham/RF-EMF exposure period. In experiments where the time-course of hsp27 phosphorylation was determined, ^{32}P-orthphosphate was present in cell cultures during the whole post-exposure incubation period (2 or 5 hours). In some experiments, p38MAPK inhibitor SB203580 (2 uM = IC_{50}) was present in cell cultures during the 1-hour sham/RF-EMF exposure.

Western blots

Blots of the 2D-gels and 1D-gels (standard SDS-PAGE) were prepared immediately after electrophoresis. Proteins were blotted on PVDF membranes (Bio-Rad, UK) using transfer buffer (25 mM Tris, 192 mM glycine, 20% MeOH, and 0.1% SDS) and APBiotech Novablot semi-dry blotting apparatus (0.8 mA/cm^2 for 45 min). Membranes were blocked o/n at + 4 °C in 0.1 M Tris-HCl pH 7.4 containing 5% non-fat dry milk. Hsp27 and p38MAPK were detected in the membranes using appropriate ImmunoCruz goat polyclonal antibodies (dilution 1:50) and alkaline phosphatase conjugated ImmunoCruz anti-Goat IgG (dilution 1:1000) and enhanced chemiluminescence (ECL) staining kit (Pierce, UK).

Immunoprecipitation

Cells were disrupted in ice-cold RIPA buffer. The lysates were centrifuged $10\,000 \times g$ for 10 min at + 4 °C to remove cellular debris. 230 ug of protein (Lowry-Ciocalteau method) from sham and irradiated lysates was placed in Eppendorf tubes and pre-cleared

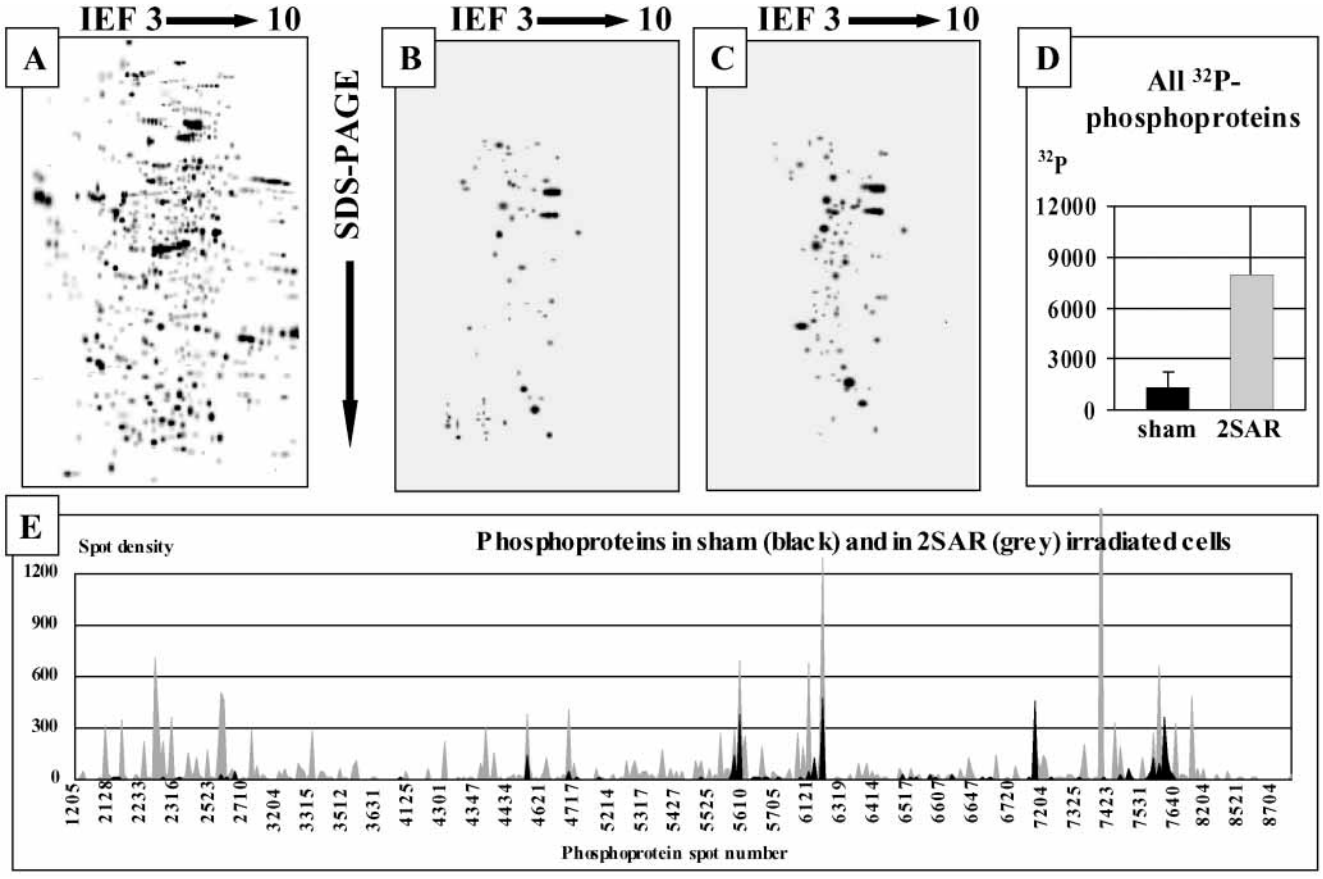

Fig. 2 (**A**) Map of proteins, isolated from EA.hy926 cells, generated with PDQuest using four separate silver-stained 2D-gels. (**B**) Representative autoradiogram (out of four) of phosphoprotein distribution in sham cells. (**C**) Representative autoradiogram (out of four) of phosphoprotein distribution in RF-EMF exposed cells. (**D**) Total content of ^{32}P-phosphoproteins that were generated during the 1 h sham/RF-EMF exposure. Arbitrary density units obtained by densitometric analysis (PDQuest). Values are mean ± SD of four sham (*black bars*) and exposed (*gray bars*) samples. (**E**) Densitometric analysis of ^{32}P-phosphoprotein spots from 2D-autoradiograms from sham (*black peaks*) and exposed (*gray peaks*) cells (average values of four repeats). Arbitrary density units obtained with PDQuest.

with 2.3 ug goat IgG (ImmunoCruz, USA) and 20 ul of rec-Protein G Sepharose 4B (Zymed, USA) at + 4 °C on a shaker for 30 min. Thereafter, rec-Protein G Sepharose 4B was removed by centrifugation, and hsp27 was immunoprecipitated with 2 ug of anti-hsp27 antibody (ImmunoCruz, USA) and 20 ul of rec-Protein G Sepharose 4B, overnight at 4 °C on a shaker. The next day, rec-Protein G Sepharose 4B beads were pelleted, washed 4 times with RIPA buffer, suspended in the SDS-PAGE sample buffer, cooked, and resolved using 8% SDS-PAGE gel. The X-ray films were exposed to dried gels to detect ^{32}P-hsp27.

Results and Discussion

Semi-confluent cultures of EA.hy926 cells (Fig. 1A) were exposed to 900 MHz GSM mobile phone radiation in a specially constructed chamber (Fig. 1B and 1C) (Jokela et al., 2000). Immediately after irradiation, cells were harvested, lysed, and proteins analyzed as described below. In experiments examining the time-course of protein expression or phosphorylation, cells were harvested at different time-intervals after the end of sham/RF-EMF exposure.

Using PDQuest software (Bio-Rad, UK), over 1 200 different protein spots were identified in silver-stained 2D-gels (Fig. 2A). Among them, 110 phosphoproteins were detected in sham cells (Fig. 2B). After RF-EMF exposure, the number of phosphoproteins increased to 372 (Fig. 2C), corresponding a more than 3-fold increase in ^{32}P incorporation (Fig. 2D). PDQuest analysis of 2D-autoradiograms of ^{32}P-phosphoproteins (Fig. 2E) revealed that 43 of the phosphoproteins were present in both sham and exposed cells (changes in phosphorylation levels among these phosphoproteins were also detected). 67 phosphoproteins were present only in sham cells, which suggests that they were dephosphorylated during the 1-hour exposure. 324 phosphoproteins appeared *de novo* in exposed cells only. However, when analyzing changes in protein phosphorylation, it is necessary to remember that the identification of phosphoproteins is difficult when based only on their mol-

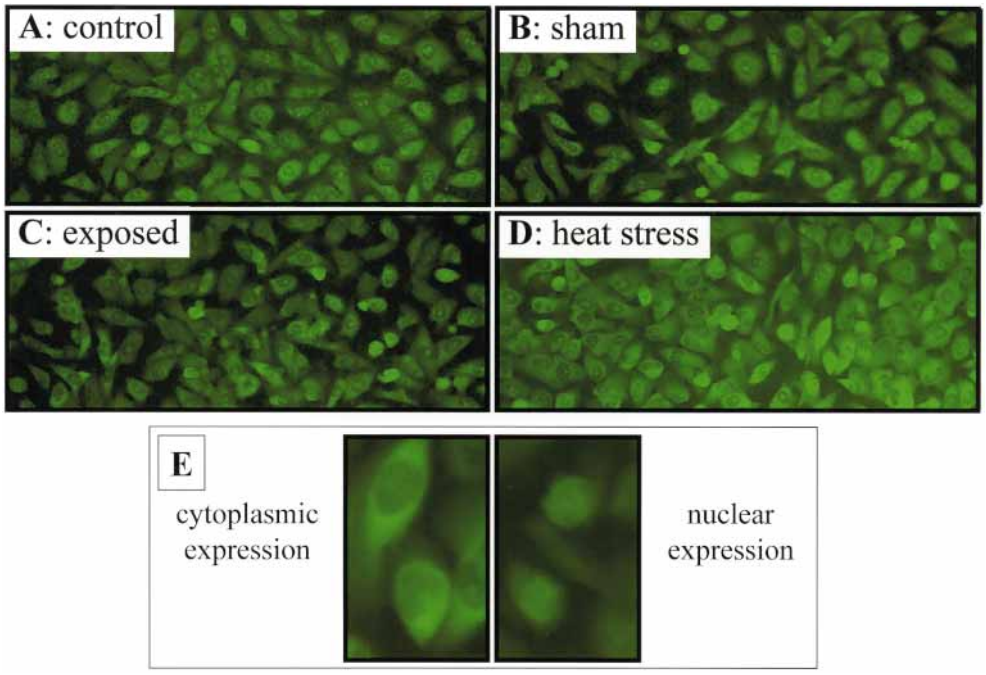

Fig. 3 Detection of hsp27 expression by indirect immunofluorescence staining. Cells were grown on microscopic glass cover-slides placed on the bottom of glass petri dishes. Cell seeding and growth conditions were the same as in experiments where protein lysates were harvested. (**A**) Control cells (cells grown in cell culture incubator). (**B**) Sham exposed cells stained immediately after 1-hour exposure. (**C**) Cells exposed for 1 hour to RF-EMF and stained immediately after the exposure. (**D**) Heat-shock exposed cells (temperature of + 43 °C for 3 hours in cell culture incubator) and stained immediately after the 3-hour heat-shock-exposure period. (**E**) Hsp27 expression was detected either in cytoplasm or in nucleus. For explanation see text.

ecular weight and pI. Changes in phosphorylation may alter the pI of a protein and thus, during the course of isoelectrofocusing, the phosphoprotein will localise in another spot than the non-phosphorylated or less-phosphorylated form of the same protein. Therefore, to correctly assign phosphorylation changes of phosphoproteins, identification of these phosphoproteins using mass spectrometry is necessary (experiments are underway, and results will be reported at later time). However, although the particular identity of phosphoprotein spots remains unknown, the 3-fold increase in incorporation of the ^{32}P-label and the increase in the number of phosphorylated protein spots clearly suggest that a variety of cellular signaling pathways of EA.hy926 endothelial cells responded to the non-thermal RF-EMF irradiation.

Hsp27 has been identified as one of the phosphoproteins that were present in phosphorylated form in both sham and exposed cells. This stress protein is continuously expressed in the EA.hy926 endothelial cell line (Wagner et al., 1999). Immunofluorescence staining revealed that some of EA.hy926 cells, in control and in sham cultures, express high levels of hsp27 (Fig. 3A and 3B). The number of hsp27 cells increased in cells exposed to RF-EMF (Fig. 3C). Heat shock was an even more potent inducer of hsp27 expression (Fig. 3D). hsp27 was detected either in cytoplasm or in the nucleus (Fig. 3E), and at this point, it is still unclear whether hsp27 expression in any of the cellular compartments was affected by RF-EMF exposure (data not shown). 2D-electrophoretic separation of protein lysates obtained from sham and exposed cells, followed by Western blotting, revealed that, in EA.hy926 cells, hsp27 was expressed as two molecular species, each of 27 kDa molecular weight but with different isoelectric points (pI = 5.7 and pI = 6.1) (Fig. 4A). Labeling of phosphoproteins with ^{32}P-orthophosphate allowed us to deduct that the $hsp27_{pI=5.7}$ isoform was phosphorylated in sham cell cultures. Following exposure to RF-EMF the size of the ^{32}P-phosphorylated $hsp27_{pI=5.7}$-spot increased. There are three possible explanations for this observation. First, the non-phosphorylated molecules of $hsp27_{pI=6.1}$ underwent phosphorylation in response to RF-EMF causing a shift of their pI to 5.7 value and co-localization of the protein molecules to $hsp27_{pI=5.7}$-spot. Second, RF-EMF exposure might have caused *de novo* synthesis of hsp27 which was then phosphorylated and co-localized in the $hsp27_{pI=5.7}$-spot. Third, it might be possible that the phosphorylation of the already phosphorylated molecules of $hsp27_{pI=5.7}$ increased but the experimental methods of this study did not allow to test this possibility. The first and the second explanation, however, appear more likely. Phosphorylation of hsp27 has been shown as an activation mechanism of hsp27 in endothelial cells (Groblewski et al., 1997; Huot et al., 1997). Also, this study presents evidence (see below) showing that RF-EMF irradiation causes transient increase in expression level of hsp27 protein (Fig. 5A). The $hsp27_{pI=6.1}$-spot remained, as expected, non-phosphorylated in sham and in exposed cells.

The increase in phosphorylation of hsp27 during the 1-hour irradiation period was confirmed by immunoprecipitation (Fig. 4B). The phosphorylation of hsp27 induced by RF-EMF exposure was abolished in the presence of SB203580, a specific inhibitor of p38MAPK (Fig. 4C) (Lee et al., 1999). Interestingly, RF-EMF exposure also appeared to transiently increase expression

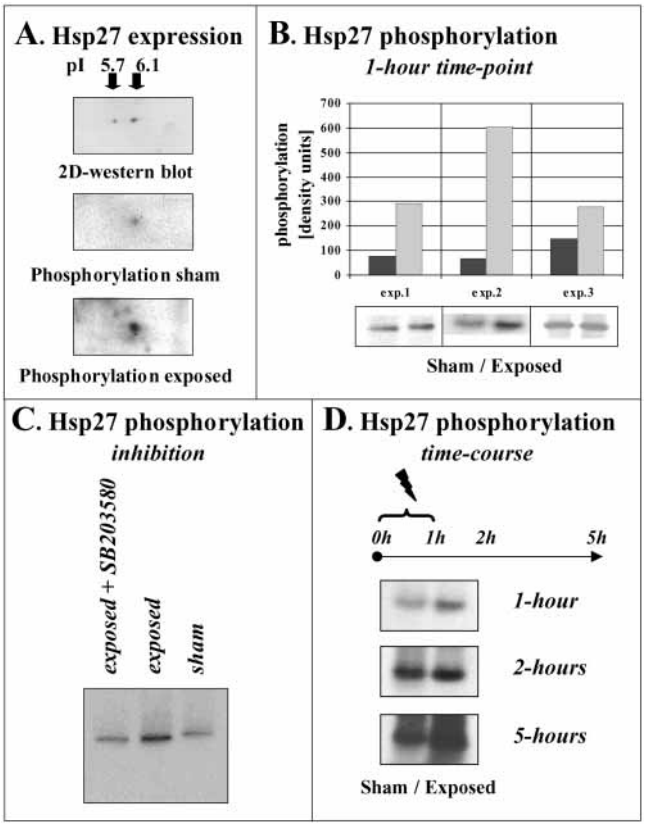

Fig. 4 (**A**) Hsp27 detected by Western blot as two separate spots (*top*) and appearance of ^{32}P-hsp27 in autoradiograms – from sham (*middle*) and RF-EMF (*bottom*) cells. (**B**) Densitometric analysis (sham: *black*; exposed: *gray*) and corresponding images from the autoradiograms of SDS-PAGE separated immunoprecipitated ^{32}P-hsp27. Cell lysates were prepared immediately after the 1-hour sham/RF-EMF exposure. (**C**) SB203580-induced inhibition of RF-EMF exposure-induced hsp27 phosphorylation. (**D**) Autoradiograms of ^{32}P-hsp27 immunoprecipitated from cells at 1-, 2- and 5-hours after the start of sham/irradiation.

of p38MAPK-kinase (Fig. 5B), which is indirectly (via kinases MAPKAPK-2/3 or PRAK) involved in phosphorylation of hsp27 (Ono and Han, 2000).

The increase in hsp27 phosphorylation induced by RF-EMF exposure was short-lasting, and the differences between sham and exposed cells were no longer detected in cell lysates from cells harvested 1 h and 4 h after the end of RF-EMF irradiation period (Fig. 4D). Taken together, the kinetics of the observed induction of hsp27 phosphorylation in response to RF-EMF (external stress) agrees with the published evidence showing that phosphorylation of hsp27 is a well-established mechanism of cell response to broad variety of stimuli (Kato et al., 1994; Rogalla et al., 1999). Also, the short-lasting increase in hsp27 phosphorylation, in response to RF-EMF, is similar to the behavior of this protein in response to other stress stimuli (Groblewski et al., 1997; Huot et al., 1997; Kato et al., 1994; Rogalla et al., 1999). Therefore, the observed changes in expression and phosphorylation of hsp27 and in expression of p38MAPK suggest that EA.hy926 cells recognize mobile phone radiation as a stress factor and, in response, activate the p38MAPK/hsp27 stress signal transduction pathway. Thus, our results also show that RF-EMF radiation is a new addition to the list of external stress factors that are able to activate cellular stress kinases.

Changes in protein phosphorylation and activation of p38MAPK/hsp27 stress response pathway observed in this study agree with earlier studies suggesting that mobile phone RF-EMF radiation induces cellular stress response at non-thermal power level. *In vitro*, Cleary et al. (1997) claimed that RF-EMF exposure has no effect on stress proteins. However, because identification of stress proteins was based solely on their molecular weight in liquid chromatography, the exact identity of proteins claimed to be stress proteins remains unclear. Fritze et al. (1997b), using a rat model, have shown an increase in expression of stress protein hsp70 in brains of animals exposed for 4 h to RF-EMF (890–915 MHz) at SAR of 1.5 W/kg. Daniells et al. (1998) and de Pomerai et al. (2000) have shown that *in vivo* irradiation of nematode worms overnight with RF-EMF (750 MHz) at SAR of 0.001 W/kg causes an increase in expression of heat shock protein. Kwee et al. (2001) have shown an induction of stress protein hsp70, but not hsp27, in cultures of transformed human epithelial amnion cells exposed for 20 min to RF-EMF (960 MHz) at SAR of 0.0021 W/kg.

Because of the known broad spectrum of physiological processes that are regulated by stress proteins (Tibbles and Woodgett, 1999) and by hsp27 in particular, it is here hypothesized that mobile phone radiation-induced activation of hsp27/p38MAPK-dependent cellular stress response might: (i) lead to the development of brain cancer due to inhibition of cell apoptosis and (ii) cause increased permeability of blood-brain barrier due to stabilization of endothelial cell stress fibers (Fig. 6).

Stress proteins are known to regulate cell apoptosis (Mehlen et al., 1996; Creagh et al., 2000; Pandey et al., 2000). RF-EMF-induced deregulation of apoptotic processes might be a risk factor for tumor development because it could lead to the survival of cells that "should" die. This notion was suggested in the hypothesis presented recently by French et al. (2001). We suggest that the apoptotic pathway regulated by hsp27/p38MAPK might be the target of RF-EMF radiation. Hsp27, stress protein shown in this study to be affected by mobile phone radiation exposure, is a member of a family of small heat shock proteins that is ubiquitously expressed in most cells and tissues under normal conditions in the form of large-molecular complexes. In response to stress, rapid phosphorylation of hsp27 on serine residues occurs (in human cells Ser-78 and Ser-82) which leads to dissociation of the large-molecular complexes into smaller units (Kato et al., 1994). Various stress factors have been indicated to be inducers of changes in expres-

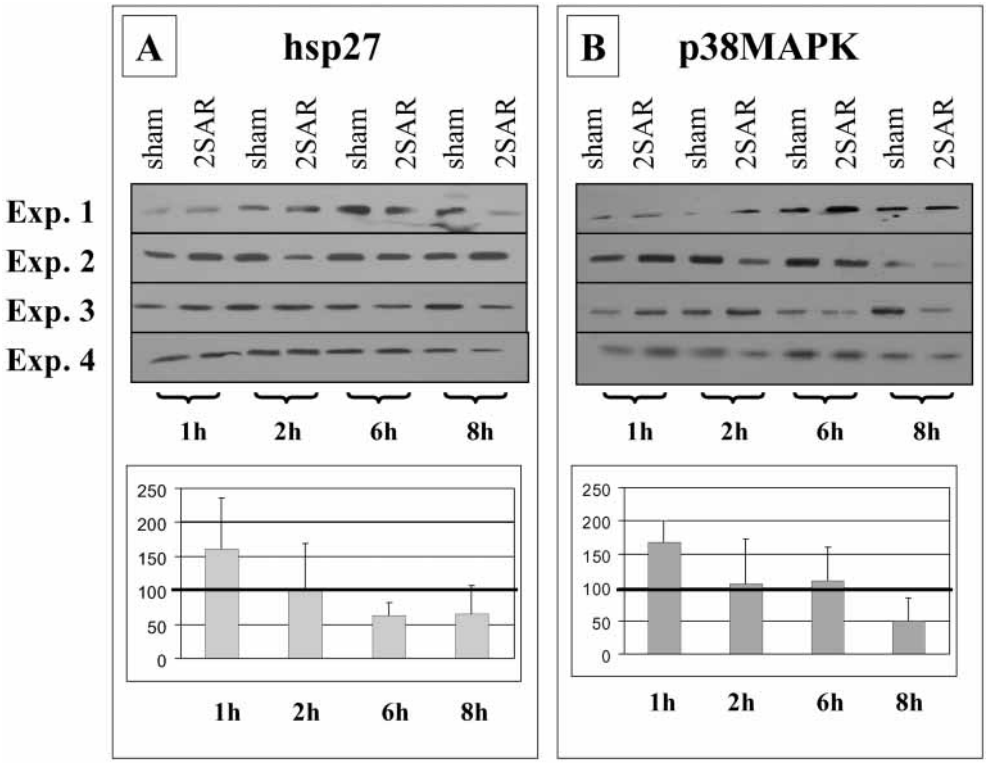

Fig. 5 Changes in expression of hsp27 (**A**) and p38MAPK (**B**) induced by RF-EMF exposure. Cells were exposed for 1 hour and collected either immediately after of irradiation (1 h-time-point) or 2 h, 6 h, and 8 h from the start of irradiation. Four separate experiments were performed. *Upper parts* of figures **A** and **B** show expression levels of hsp27 and p38MAPK detected using SDS-PAGE and Western blot. The same amounts of protein were loaded on every lane of the SDS-PAGE. *Lower graphs* show results of densitometric analysis of the changes in hsp27 and p38MAPK expression. The expression levels in sham samples were set as 100% separately for each time-point (bold vertical line). *Bars* depict protein expression in irradiated samples as a percent of the sham expression level for the given time-point.

sion (accumulation) and/or phosphorylation (activity) of hsp27 (Deli et al., 1995; Ito et al., 1995; Garrido et al., 1997; Huot et al., 1997; Tibbles and Woodgett, 1999). Activated (phosphorylated) hsp27 has been shown to inhibit apoptosis by forming a complex with the apoptosome (complex of Apaf-1 protein, pro-caspase-9 and cytochrome *c*) or some of its components and preventing proteolytic activation of pro-caspase-9 into active form of caspase-9 (Pandey et al., 2000; Concannon et al., 2001). This, in turn, prevents activation of pro-caspase-3 which, in order to become active, has to be proteolytically cleaved by caspase-9. Thus, induction of the increased expression and phosphorylation of hsp27 by the RF-EMF exposure might lead to inhibition of the apoptotic pathway that involves apoptosome and caspase-3. This event, when occurring in RF-EMF exposed brain cells that underwent either spontaneous or external factor-induced transformation/damage, could support survival of the transformed/damaged cells which, in favorable circumstances, could help clonal expansion of the transformed/damaged cells – a prerequisite for the tumor development. Furthermore, hsp27 in particular was shown to be responsible for the induction of resistance of tumor cells to death induced by anti-cancer drugs (Huot et al., 1996; Garrido et al., 1997). Thus, it appears possible that RF-EMF induced changes in hsp27 phosphorylation/expression might affect not only tumor development but also its drug-resistance.

Induction of the increase of the permeability of blood-brain barrier by RF-EMF exposure, which has been suggested by some animal and *in vitro* studies, is one of the controversial health issues that came up in relation to the use of mobile phones. It has already been established that, at thermal levels of exposure, microwave radiation causes increase in the permeability of blood-brain barrier (for review see Jokela et al., 1999; The Royal Society of Canada Report, 1999; Stewart Report, 2000; Zmirou Report, 2001). However, the effect of non-thermal RF-EMF exposure on blood-brain barrier is still unclear. Some studies suggest that mobile phone radiation, at non-thermal exposure levels, increases permeability of blood-brain barrier *in vivo* (Salford et al., 1994) and *in vitro* (Schirmacher et al., 2000), whereas others report the lack of such effects (Fritze et al., 1997a; Tsurita et al., 2000). However, the no-effect claimed by Fritze et al. (1997a) is not so straight forward as suggested by the authors because they reported induction of stress response and increased permeability of the blood-brain barrier immediately after irradiation. This effect was short lasting and was, therefore, considered by the authors as insignificant. Also, the blood-brain barrier response to repeated exposures to mobile phone radiation remains unclear because the effect of repeated exposures was not examined. The increased blood-brain barrier permeability due to an increase of endothelial pinocytosis was suggested by Neubauer et al. (1990) who demonstrated an increase in pinocytosis of cerebral cortex capillaries that were exposed to 2.45 GHz microwave radiation. Finally, the recently reported study by Töre et al. (2001) has shown that 2-hour exposure of rats to RF-

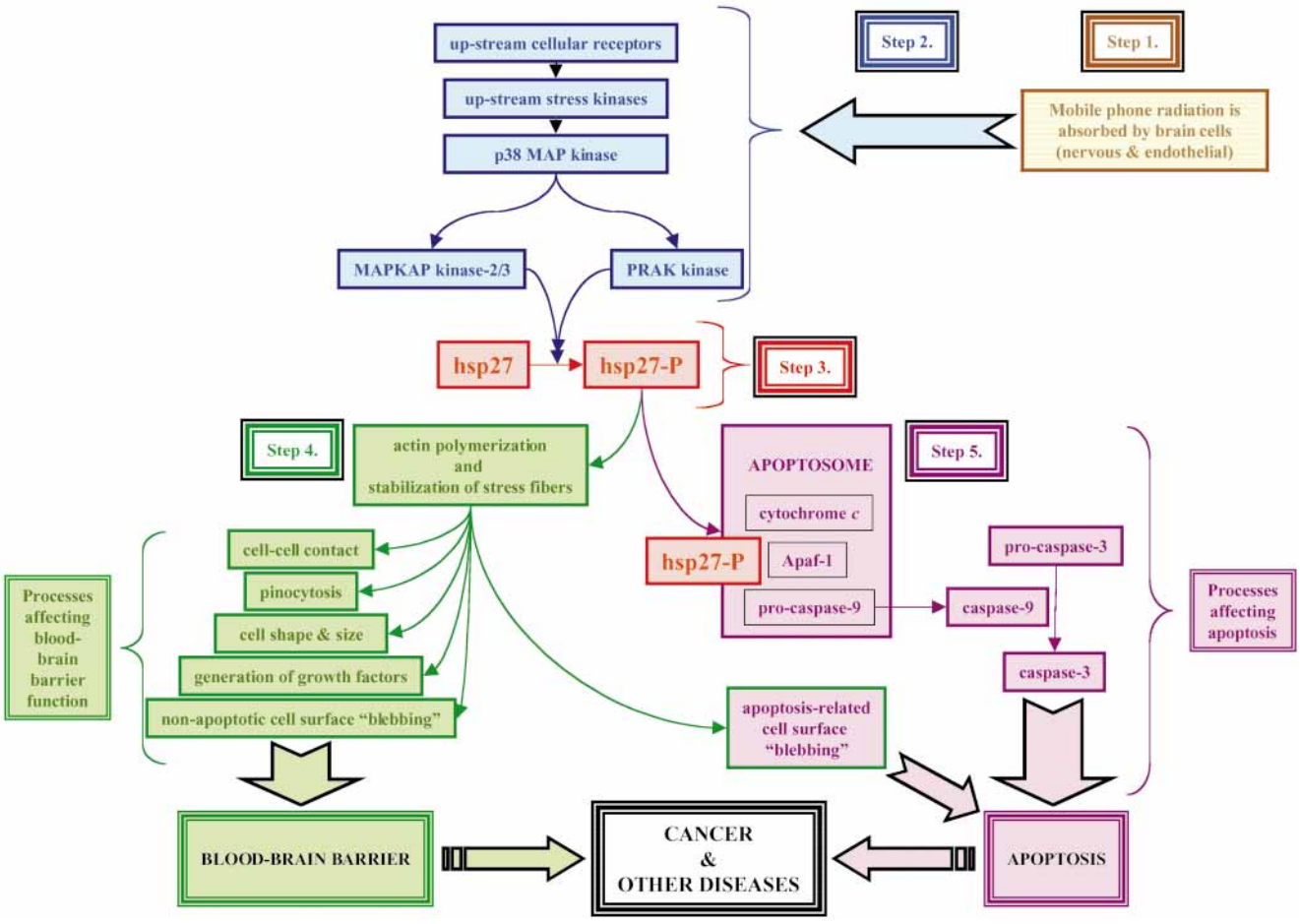

Fig. 6 Hypothetical flow of events that may occur in brain cells (nervous and endothelial) in response to mobile phone radiation: **Step 1**. Mobile phone radiation is absorbed by brain tissue – nerve cells and endothelial cells lining capillary blood vessels. **Step 2**. Mobile phone radiation, by a yet unidentified biophysical mechanism, activates directly or indirectly cellular stress kinase(s). Activation of stress response is caused either by activation of p38 MAP kinase alone or also some other kinase(s) located upstream/downstream of p38 MAP kinase. **Step 3**. The mobile phone radiation-induced kinase(s) activation leads to phosphorylation of hsp27 (hsp27-P). Simultaneously, an increase in the expression of hsp27 occurs which might have an additional, potentiating, effect by providing more hsp27 molecules for the phosphorylation-activation. **Step 4**. The phosphorylated form of hsp27 stabilizes endothelial cell stress fibers and, in conjunction with estrogen, alters generation/secretion of bFGF. This causes the increase in blood-brain barrier permeability, due to the occurrence of one or several of the following processes:changes in endothelial cell shape and formation of gaps between cells; increased (abnormally high?) pinocytosis through the endothelial monolayer; obstruction of capillary blood flow by bleb formation (and shedding?) on the lumen surface of endothelial cells – this event could increase blood pressure locally and help to force large molecules to pass though the endothelial monolayer; in females, possible formation of gaps between endothelial cells caused by induction of de-differentiation and proliferation of endothelial cells, in endocrine fashion, by endothelium-released bFGF. Endothelial cells stimulated to proliferate will round-up and gaps between the cells will be formed. **Step 5**. The phosphorylated form of hsp27 forms a complex with apoptosome, or some of its components, which prevents activation of pro-caspase-9 and subsequently prevents activation of pro-caspase-3 and inhibition of caspase-3-dependent apoptosis pathway. Thus, mobile phone radiation-induced phosphorylation of hsp27 in cells that are in the process of execution of caspase-9/caspase-3 dependent apoptosis may prevent the destruction of the unwanted damaged/transformed cells.

EMF (900 MHz) at SAR of 2 W/kg (averaged over the brain) causes increase in the permeability of blood-brain barrier. However, the molecular mechanism and the cellular signaling pathways involved in the induction of blood-brain barrier permeability are still unknown. We propose that the induction of hsp27 phosphorylation and increased expression by RF-EMF exposure shown in this study to occur *in vitro* in human endothelial cells might be the molecular signaling event that triggers the cascade of events leading to the increase in blood-brain barrier permeability. Phosphorylated hsp27 has been shown to stabilize endothelial cell stress fibers due to the increased actin polymerization (for review see Landry and Huot, 1995). The stabilization of stress fibers was shown to cause several alterations to endothelial cell physiology: (i) cell shrinkage and the opening of spaces between cells (Landry and Huot, 1995; Piotrowicz and Levin, 1997), (ii) increase in the permeability of the en-

dothelial monolayer (Deli et al., 1995), (iii) increase in pinocytosis (Lavoie et al., 1993), (iv) formation of apoptosis-unrelated blebs on the surface of endothelial cells which may obstruct blood flow through capillary vessels (Becker and Ambrosio, 1987), (v) stronger responsiveness of endothelial cells to estrogen and, when stimulated by this hormone, secretion of larger than normal amounts of basic fibroblast growth factor (bFGF) (Piotrowicz et al., 1997) which could, endocrinologically, stimulate de-differentiation and proliferation of endothelial cells leading to the – associated with proliferative state – cell shrinkage and unveiling of the basal membrane. Occurrence of these events in brain capillary endothelial cells could lead to de-regulation of the mechanisms controlling permeability of the blood-brain barrier. Furthermore, in addition to blood-brain barrier effects, the stabilization of stress fibers in endothelial cells may affect the apoptotic process – it has been shown that the apoptosis-related cell-surface blebbing is prevented by the stabilized stress fibers (Huot et al., 1998).

The proposed hypothetical molecular mechanism for a possible role of mobile phone radiation in the development of brain cancer and in increasing the permeability of the blood-brain barrier (Fig. 6), although a hypothesis, is reasonably supported by the evidence concerning both the effects of microwaves on stress response and the effects of hsp27 (increased expression and activity) on cell physiology. Proving or disproving this hypothesis using *in vitro* and *in vivo* models will provide evidence to either support or to discredit the existence of some of the potential health risks suggested to be associated with the use of mobile phones.

The recently published hypothesis of French et al. (2001) regarding the possible induction of abnormally high levels of stress proteins in cells due to chronic/frequent exposure to mobile phone radiation still requires experimental confirmation that repeated exposure to RF-EMF radiation could cause such an increase. On the other hand, the hypothetical mechanism of the mobile radiation effect on the brain, proposed by us, relies on the single-exposure-induced transient increases in hsp27 phosphorylation and expression. We suggest that the transient effects, induced by repeated exposures, might, by timing coincidence, lead to the survival of damaged/transformed cells and temporarily increase the permeability of the blood-brain barrier. Repeated occurrence of these events (on a daily basis) over a long period of time (years) could become a health hazard due to a possible accumulation of brain tissue damage. Furthermore, our hypothesis suggests that, apart from RF-EMF, other cell-damaging factors might play a co-participating role in the tumor development caused by mobile phone radiation.

Finally, in addition to the p38MAPK/hsp27 stress pathway-induced effects, the extent of the global change of the pattern of protein phosphorylation observed in our study suggests that it is likely that multiple signal transduction pathways might be affected by RF-EMF exposure. Identification of these pathways will help to determine the extent of biological effects induced by RF-EMF exposure. Importantly, cellular effects observed in this study were induced by RF-EMF irradiation at non-thermal levels, with SAR values set at the highest level allowed by the European safety limits. This suggests that the presently allowed radiation emission levels for the mobile phones, although low, might be sufficient to induce biological effects. However, determination of whether these effects might cause any significant health effects requires further investigation.

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