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Exposure to Global System for Mobile Communication (GSM) Cellular Phone Radiofrequency Alters Gene Expression, Proliferation, and Morphology of Human Skin Fibroblasts

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Human skin fibroblasts were exposed to global system for mobile communication (GSM) cellular phone radiofrequency for 1 h. GSM exposure induced alterations in cell morphology and increased the expression of mitogenic signal transduction genes (e.g., MAP kinase kinase 3, G_2/mitotic-specific cyclin G_1), cell growth inhibitors (e.g., transforming growth factor-β), and genes controlling apoptosis (e.g., bax). A significant increase in DNA synthesis and intracellular mitogenic second messenger formation matched the high expression of MAP kinase family genes. These findings show that these electromagnetic fields have significant biological effects on human skin fibroblasts.

Key words: Nonionizing radiation; Gene expression; Cell growth; Cellular phone

With the widespread use of wireless communication technology, concern over potential health hazards of long-term exposure from cellular telephones has been steadily increasing. Previous studies have reported conflicting results on the effects of electromagnetic fields on the central nervous system and cancer-related biological processes (1–5). Considering that the epidermis is the human tissue closest to the telephone, we studied the effects of global system for mobile communication (GSM) radiofrequency exposure in human skin fibroblasts. We evaluated changes in cell morphology by scanning electron microscopy and we determined whether GSM frequency elicited changes in gene expression. Changes in gene expression were studied in association with measurements of intracellular mitogenic second messengers generated at the plasma membrane, diacylglycerol and inositol phosphates, and of DNA synthesis, evaluated by thymidine incorporation.

MATERIALS AND METHODS

Cell Lines

The cell line "Detroit 550" (normal human skin fibroblasts) was from the Zooprophylactic Institute of Lombardia and Emilia, Brescia, Italy. The cell line was cultured as recommended by the Zooprophylactic Institute. Briefly, cells were cultured in minimum essential medium in Earle Salts (GIBCO BRL, Milano, Italy) supplemented with 10% fetal calf serum (Mascia Brunelli, Milano, Italy), 100 U/ml penicillin, and 100 U/ml streptomycin (Mascia Brunelli) at 37°C in an incubator for mammalian cell cultures (Heraeus, Milano, Italy), in a controlled atmosphere of 5% CO_2 and 95% humidity. To prevent changes in the optimum pH range (6.9–7.4), when cells were removed from the CO_2 incubator for routine splitting or exposure experiments the culture medium was also supplemented with the organic buffer HEPES (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]) at a concentration of 25 mM (6). Cells were split once a week, or when necessary, at a density 1:4–1:2, using a trypsin/EDTA solution (Sigma Chemical Co., St. Louis, MO).

Exposure to Radiofrequency Used in Cellular Phone Communications

Exponentially growing cells in six-well culture plates (2) were placed outside of the CO_2 incubator for 1 h before exposure. It should be noted that established mammalian cell lines, such as Detroit 550 fibroblasts, cultured in appropriately buffered medium, are routinely kept outside the CO_2 incubator for 1–2 h during splitting, harvesting, or other procedures without suffering cold or pH shock (6). Exposure consisted of placing culture plates for 1 h above a commercial cellular telephone (frequency 902.4 MHz) switched on the answering mode, in communication with a second telephone located in a distant room, close to a voice source, so as to reproduce an ordinary telephonic communication. The average specific absorption rate (SAR) was 0.6 W/kg. According to current European law, the maximum value for electric field intensity was 9–10 V/min at 1 cm; maximum value for radiated power density was 1 W/m^2, much lower than the currently admitted standard value (i.e., 4 W/m^2). The electric field was evaluated by an Alphalab Inc. Trifield™ meter (Ghent, NY, USA). Adequate ventilation was provided. The temperature at the plate level was monitored with a digital thermometer

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(Shimazu Scientific Co., Osaka, Japan) and no significant variation was recorded throughout the experiments.

Control (sham-exposed) cultures received the same treatment (i.e., permanence outside the CO_2 incubator) and were placed in a nearby room above an identical cellular telephone in the switched off mode, without telephonic communication and radiofrequency. The temperature (26°C) and humidity conditions (60%) were the same in the two rooms as measured by conventional instrumentation (Oregon Sci., USA). The rooms for the experiments were ordinary reading rooms with no particular electric equipment or apparatuses. They are located on the first floor of the Department of Anatomy of the University of Firenze, at a distance of about 500 m from the nearest base station of the cellular telephone company (Telecom Italia). It is worth noting that this is a residential suburban area with no major industries or power lines. In order to rule out artifacts due to cell culture permanence outside the incubator, additional control plates were kept inside the incubator during the experiments.

cDNA Expression Arrays

We used an Atlas™ Human Array Trial kit (CLONTECH, Mountainveiw, CA) that includes membranes containing 82 human cDNAs, 9 housekeeping control cDNAs, and negative controls immobilized in duplicate dots on a nylon membrane. Total RNA was extracted from in vitro cells immediately after exposure with an RNeasy® Mini Kit (QIAGEN, Valencia, CA, USA). The complex ^{32}P-labeled first-strand cDNA probes were synthesized and purified according to the protocol provided in the Atlas cDNA Expression Arrays User Manual (CLONTECH). Quantitation of hybridization intensity of each dot doublet on the array was performed as follows. The autoradiography of film images was scanned and stored as TIFF files using a computer scanner. The DNA spots were then analyzed and compared by Scion Image image processing and analysis program (Scion Corporation, Frederick, MD, USA). Any gene determined by a DNA spot intensity with a difference in expression of greater that 1.5-fold between control and exposed cultures was selected (reported in Table 1).

Study of Cell Proliferation

Following a well-established method to evaluate proliferation in cells exposed to electromagnetic fields (7), DNA synthesis was monitored by incorporation of [^{3}H]thymidine (Amersham, Arlington Heights, IL, USA; 1 μCi/ml, specific activity 24.0 Ci/mmol). Cells were seeded in tissue culture plates at a density of 7×10^4 cells per well. Radiofrequency-exposed and control cells were pulse-labeled with [^{3}H]thymidine as described previously (8), and ^{3}H radioactivity was measured by liquid scintillation spectrometry. Each experiment was performed in triplicate samples. In a typical experiment a concentration of approximately 1×10^6 cells/ml was used, and incorporation of radioactive precursor was as follows: 162,900 ± 6730 (cpm ± SE, $n = 6$).

Second Messenger Measurements

Cells were seeded in tissue culture plates at a density of 5×10^5 per well. The level of second messengers was determined in cells prelabeled to equilibrium with 10 μCi/ml of [^{3}H]glycerol (Amersham; 10 μCi/ml, specific activity 1.00 Ci/mmol) or with 10 μCi/ml of [^{3}H]inositol (Amersham; 10 μCi/ml, specific activity 117 Ci/mmol) for 24 h. Immediately after each experiment, a modified Folch extraction was performed by adding ice-cold methanol and chloroform (1:1, v/v) (9,10). Diacylglycerol, phospholipids, and inositol phosphates were extracted and separated as described previously (10). Results of diacylglycerol and inositol phosphate measurements were expressed as fold variation in each analyzed metabolite over that without exposure. In a typical experiment, a concentration of approximately 1×10^6 cells/ml was used, and incorporation of radioactive labeling was as follows: diacylglycerol 1430 ± 67; glycerol-containing phospholipids, 2,644,300 ± 9600 (cpm ± SE, $n = 6$, of radioactivity associated with diacylglycerol or phospholipids spots on TLC plate); inositol phosphates, 4350 ± 120; inositol-containing phospholipids, 43,260 ± 1540 [cpm ± SE, $n = 6$, of radioactivity recovered in the total inositol lipid fraction, and of an aliquot (100 μl) of the organic phase of modified Folch extraction]. In order to minimize error due to variability in cell number, labeling procedure, and extraction efficiency, the ratios diacylglycerol/phospholipids and inositol phosphates/phosphoinositides were taken into account in making calculations (8–10).

Studies of Cell Morphology

Cell morphology was examined by scanning electron microscopy immediately after exposure. Cells, cultured for this purpose on small glass dishes, were fixed in 2% glutaraldehyde in phosphate buffer (pH 7.4), and then postfixed in 1% osmium tetroxide. After this, cells were dehydrated in graded ethanol and dried in a critical point dryer. Specimens were then coated with 10% gold/90% palladium, and observed using a Cambridge Stereoscan 100 microscope, at an accelerating voltage between 15 and 25 kV. To quantify the morphological changes we followed a well-assessed procedure (11). Specimens were observed along their entire surface by scanning electron microscopy at a constant magnification of ×1000. In each specimen, five square fields of 2500 μm^2 (50×50 μm) were randomly chosen and photographed. The pictures were then evaluated by an image analysis system (Quantimet 600, Leica, Cambridge, UK), determining the percentage of surface lined with altered cells.

RESULTS

Effects of GSM Radiofrequency Exposure on Cell Morphology

By scanning electron microscopy, the control cells (sham exposed or those kept in the incubator) were isolated or gathered in small groups (Fig. 1A–C). The cells were dome shaped and showed a rather smooth plasmalemma, sometimes with little globular masses, probably secretory granules. The basal contour was generally expanded in spindle-like expansions or in a velamentous border, probably expression of cell adhesion. The cellular dimensions varied greatly, but the large majority showed a diameter near 2–2.5 μm. In exposed cells, the plasmalemma sometimes had an irregular appearance, some bulging in irregular masses, some deeply infolded

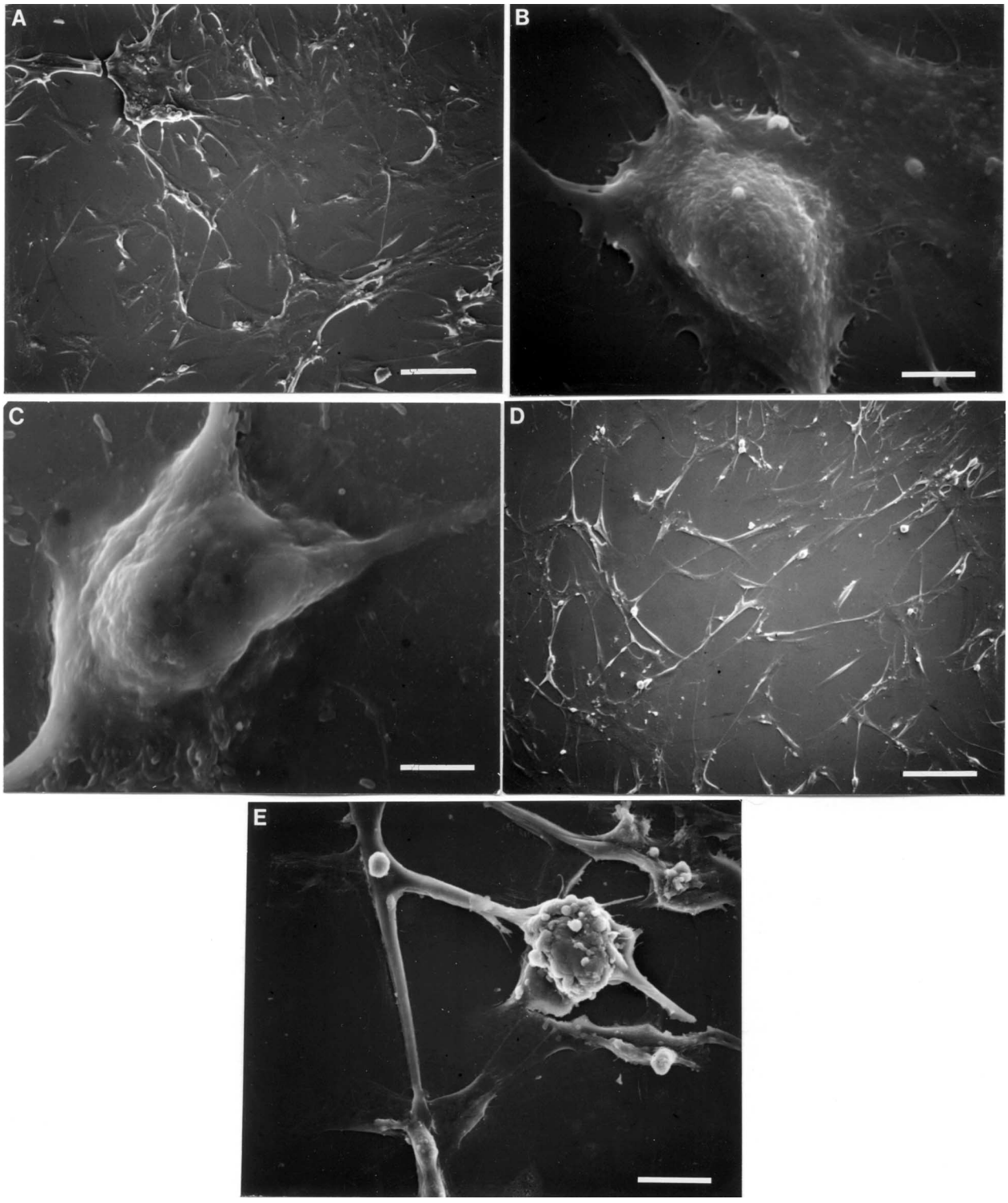


Figure 1. Scanning electron microscopy of control and GSM radiofrequency-exposed cells. (A) Control cells. A group of large cells shows an irregularly polygonal outline and a velamentous shape. Each cell adheres to the others by means of branched filaments. Sham-exposed cells and cells kept in the incubator show quite the same appearance. ×150. Bar = 100 µm. (B) Sham-exposed cells. A dome-shaped cell with some globular secretory blebs adheres to the plate by means of its velamentous basal cytoplasm. ×2700. Bar = 5 µm. (C) Unexposed cells (kept in the incubator). This cell shows a roundish body, regular plasmalemma, and signs of adhesion to the substrate. ×2800. Bar = 5 µm. (D) Exposed cells. A group of small, elongated cells shows scarce and long filaments. ×150. Bar = 100 µm. (E) Exposed cells. This cell shows a small body with irregularly bulging apical plasmalemma. Long, poorly branched filaments are to be noticed. ×2700. Bar = 5 µm.

(Fig. 1D–E). Cells showed a spindle-like shape with long and stiff protrusions and a smaller diameter than sham-exposed cells. Cell-to-cell adhesion signs in the basal portion were sometimes discontinued. The altered cells occupied a mean area ranging from 22.17% to 31.52% of the entire surface.

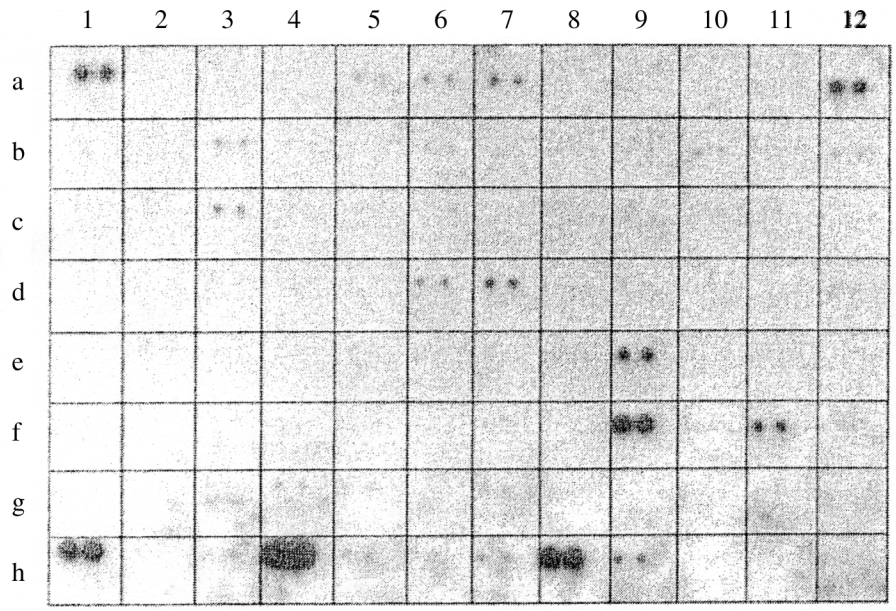
Effects of GSM Radiofrequency Exposure on Gene Expression

The probe from each Detroit 550 specimen was hybridized to a cDNA array, with 82 human cDNAs. Identification of the expressed target gene was based on a distinct position and pattern in the array (Table 1, Fig. 2). The GenBank accession number and gene/protein name were retrieved from the coordinates of hybridization signals available in the Atlas cDNA Expression Arrays User Manual (CLONTECH). Only paired DNA clones with similar signal intensities, as determined by the imaging software, were considered positive. Following an established procedure (12), we only considered the DNA spot pairs with intensity differences greater than 1.5-fold. Thus, only genes whose expression was significantly increased in exposed cells, in comparison with control cells, are reported in Table 1.

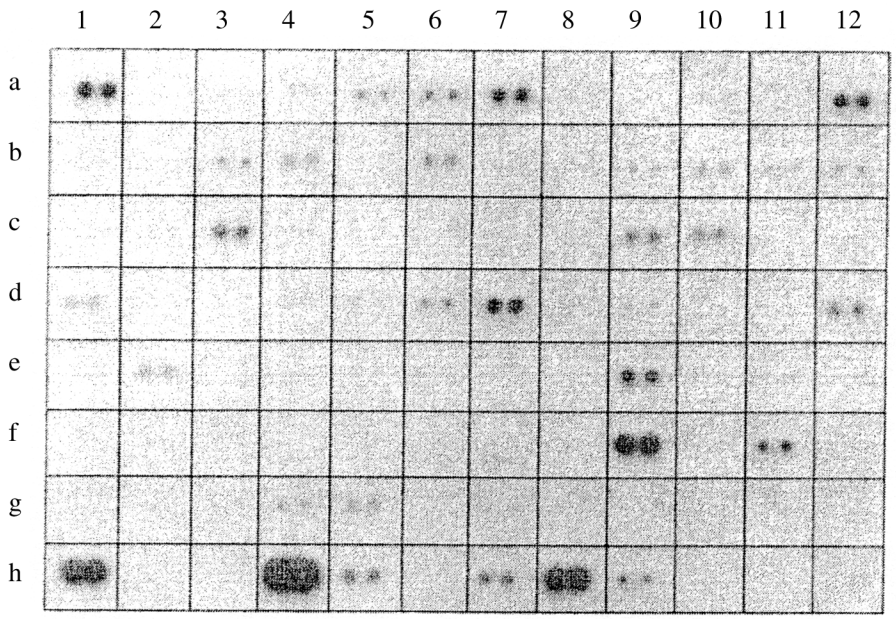
Genes that were highly expressed in exposed cells include stress response proteins such as growth arrest and DNA damage-inducible protein (GADD45); DNA damage-inducible transcript 1 (DDIT1); glutathione *S*-transferase pi (GSTP1); GST3 and ionizing radiation resistance-conferring protein + death-associated protein 3 (DAP3). We also found that cell cycle regulators were significantly highly expressed in exposed cells. Among these, transforming growth factor-β (TGF-β; TGFB); dual specificity mitogen-activated protein kinase kinase 3 (MAP kinase kinase 3); ERK activator kinase 3; MAPK/ERK kinase 3 (MEK3); cyclin-dependent kinase inhibitor 3 (CDKN3); CDK2-associated dual-specificity phosphatase (KAP); cyclin-dependent kinase interacting protein 2 (CIP2); cyclin-dependent kinase interactor 1

Table 1. Atlas Human Array Trial Kit

Gene No.	Gene Name	Coordinate	GenBank Accession No.(s)	Swiss-Prot I Accession No.(s)
Stress response proteins				
719	growth arrest & DNA-damage-inducible protein (GADD45); DNA-damage-inducible transcript 1 (DDIT1)	6B	M60974	P24522
652	glutathione *S*-transferase pi (GSTP1); GST3	9F	X08058; M24485	P09211
672	ionizing radiation resistance-conferring protein + death-associated protein 3 (DAP3)	10C	U18321 + X83544	Q13044 + P51398
Cell cycle regulators				
34	transforming growth factor-β (TGF-β; TGFB)	1D	X02812; J05114	P01137
705	dual specificity mitogen-activated protein kinase kinase 3 (MAP kinase kinase 3; MAPKK 3; MKK3); ERK activator kinase 3; MAPK/ERK kinase 3 (MEK3)	3C	L36719	P46734
876	cyclin-dependent kinase inhibitor 3 (CDKN3); CDK2-associated dual-specificity phosphatase; kinase-associated phosphatase (KAP); cyclin-dependent kinase interacting protein 2 (CIP2); cyclin-dependent kinase interactor 1 (CDI1)	4B	L25876	Q16667; Q99585
638	DNA topoisomerase II alpha (TOP2A)	5G	J04088	P11388
4	c-*myc* proto-oncogene	7D	V00568	P01106; P01107
373	apoptosis regulator bax	9C	L22474	Q07814
749	G_2/mitotic-specific cyclin G_1 (CCNG1; CYCG1)	12D	U47413	P51959
Nuclear receptors				
77	retinoic acid receptor epsilon (RAR-epsilon); retinoic acid receptor beta 2 (RAR-beta2; RARB); HAP	2E	X07282; Y00291	P10826
Cytoskeleton				
901	brain-specific tubulin alpha 1 subunit (TUBA1)	5H	K00558	P04687
49	cytoplasmic beta-actin (ACTB)	7H	X00351	P02570; Q11211; P99021; Q64316; P70514
Extracellular matrix				
C82	fibronectin precursor (FN)	7A	X02761; K00799; K02273; X00307; X00739	P02751; Q14326



Control



GSM exposed

Figure 2. Differential gene expression of control and GSM radiofrequency-exposed cells as determined by cDNA microarray analysis. The differences in gene expression were determined by comparing the paired images with Scion Image image processing and analysis program.

(CDI1); DNA topoisomerase II alpha (TOP2A); c-*myc* proto-oncogene; apoptosis regulator bax; and G_2/mitotic-specific cyclin G_1 (CCNG1; CYCG1). Nuclear receptors [retinoic acid receptor epsilon (RAR-epsilon); retinoic acid receptor beta 2 (RAR-beta 2; RARB); and (HAP)] were significantly highly expressed in exposed cells. Genes coding for cytoskeletal proteins [brain-specific tubulin alpha 1 subunit (TUBA1); and cytoplasmic beta-actin (ACTB)], or for extracellular matrix proteins [fibronectin precursor (FN)] were also highly expressed following exposure to GSM radiofrequency.

Effects of GSM Radiofrequency Exposure on DNA Synthesis and Second Messenger Formation

The products of inositol lipid metabolism, diacylglycerol and inositol phosphates, affect a number of cellular functions by activating protein kinase C and mobilizing intracellular calcium. Diacylglycerol and inositol phosphate formation in exposed cells was increased over controls (Table 2). To ascertain whether the increase in diacylglyerol and inositol phosphate was specific for the cellular phone radiofrequency exposure or was just part of increased metabolism and uptake of radioactive precursors, we measured the level of radioactivity associated with total polyphosphoinositides and found no significant difference between exposed cells and controls (Table 2). To rule out the possibility of a generalized metabolic disorder in the exposed cells, we also determined (NADP)-isocitrate dehydrogenase activity; no significant differences in this enzyme activity were observed between exposed cells and controls (data not shown). Cell proliferation in exposed cells, evaluated as DNA synthesis monitored by [^{3}H]thymidine incorporation, was significantly increased, matching the higher expression of the growth-related genes and of mitogenic messenger formation (Table 2).

DISCUSSION

In this study we have demonstrated that exposure of cultured human cells to cellular phone radiofrequencies caused morphological and functional changes. The observed effects on cell architecture could be associated with the increased expression of genes coding for structural proteins. Also, genes related to growth regulation were highly expressed in exposed cultured cells. The concomitant expression of genes promoting or inhibiting cellular growth or related to apoptosis may relate to a stress response. The increase in DNA synthesis observed in exposed cells matched the rise in diacylglycerol, the second messenger that, through protein kinase C, induces the expression of MAP kinase family genes. Whatever the mechanisms involved, further studies are warranted to clarify whether the cellular modifications described in the present study represent an health hazard in the long term.

Table 2. Levels of Thymidine, Inositol Phosphates (IPs), Diacylglycerol (DAG), and Polyphosphoinositides (PiPs) in Human Skin Fibroblasts Exposed to GSM Cellular Phone Radiofrequency

Exposure	Thymidine	IPs	DAG	PiPs
Incubator	1.00	1.00	1.00	1.00
Sham	0.97	0.99	0.98	1.01
GSM	1.43	2.73	3.19	0.99

Cell cultures were exposed to GSM cellular phone radiofrequency as described (GSM). Alternatively, cells were kept in the CO_2 incubator under normal culture conditions (Incubator), or sham exposed (Sham) (i.e., placed above a nonfunctioning cellular telephone, outside the incubator). Values obtained for IPs and DAG were normalized for the amount of radioactivity associated with the precursor phospholipid. PiPs: total polyphosphoinositides. Results shown in the table are expressed as fold variation in each analyzed metabolite over that without exposure from a representative experiment, one out of four that gave quantitatively identical results.

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