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ANNE HÖYTÖ

Cellular Responses to Mobile Phone Radiation

Proliferation, Cell Death and Related Effects

Doctoral dissertation

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ABSTRACT

The rapidly expanding use of mobile phones utilising radiofrequency (RF) radiation has raised public concern on possible health effects of RF radiation. In general, epidemiological studies and animal experiments have not provided any evidence of detrimental health effects from RF radiation at exposure levels that do not heat biological tissues. However, some *in vitro* studies have indicated that also weak RF radiation may affect some cellular functions. For example, effects on ornithine decarboxylase (ODC) activity, proliferation, programmed cell death (apoptosis), gene and protein expression, and genotoxicity have been reported. The aim of the present study was to evaluate whether RF radiation could interfere with some cellular functions such as ODC activity, proliferation, oxidative stress, and cell death. Different cell lines and different modulations of the RF signal were used to test if the effects are specific to certain cell types or modulations. Furthermore, combined exposures with other stressors were used in some of the studies to enhance the probability of detecting any possible weak effects of RF radiation. To maximize the probability of finding any effects, relatively high exposure levels up to 6 W/kg (clearly exceeding worst-case exposure scenarios of mobile phone users) were used. The exposure systems used in the study allowed cooling of the cell cultures to avoid any temperature increase that would otherwise result from the highest exposures.

Four different cell types (murine L929 fibroblast, human SH-SY5Y neuroblastoma, rat C6 glioblastoma, and rat primary astrocyte cells) were exposed to RF radiation in two different exposure chambers; waveguide chamber (frequencies 872 and 900 MHz) and transverse electromagnetic (TEM) cell (frequency 835 MHz). Cell cultures were exposed to three different types of RF signals for 1–24 h alone or in combination with another stressor. Fresh medium and serum deprivation as well as oxidative stress inducing agents were used to stress the cell cultures. After exposure, ODC activity, proliferation, reduced glutathione (GSH) levels, lipid peroxidation, caspase-3 activity, and viability of the cells were measured.

The ODC activity of primary rat astrocytes was significantly reduced after exposure to RF radiation, but ODC activity and proliferation of secondary cell lines were generally not affected. Some indications of effects on cellular apoptosis were detected, since caspase-3 activities were increased after combined exposures to stressors and RF radiation in L929 cells. A possible effect of RF radiation on lipid peroxidation in stressed SH-SY5Y cells was also observed. No effects on GSH levels or cellular viability were detected.

Primary rat astrocytes were more sensitive than secondary cell lines to the effects of RF radiation on cellular ODC activity. This is an interesting finding, since the physiology of primary cells is closer to that of intact tissues than that of transformed cells. The possible effects on apoptosis and lipid peroxidation will require confirmation in further studies. There was no evidence on modulation-specific effects of RF radiation. More studies are warranted to evaluate the effects of RF radiation on primary cells and apoptosis in stressed cells.

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Medical Subject Headings: Apoptosis; Cell Proliferation; Cellular Phone; Cells, Cultured; Electromagnetic Fields/adverse effects; Glutathione; Lipid Peroxidation; Necrosis; Ornithine Decarboxylase; Radiation, Nonionising/adverse effects; Radio Waves/adverse effects

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Kuopio, March 2008,

Anne Höytö

ABBREVIATIONS

AMA	Transformed human epithelial amnion cells
CAT	Catalase
CDMA	Code division multiple access
CHO	Chinese hamster ovary
CTLL-2	Cytolytic T lymphocytes
CW	Continuous wave
DAMPS	Digital advanced mobile phones system
DAPI	4’,6-diamino-2-phenylindole
DCF-DA	2',7'-dichlorofluorescein-diacetate
DNA	Deoxyribonucleic acid
DTX	Discontinuous transmission mode
EEG	Electroencephalogram
FACS	Fluorescent activated cell sorting
FADD	Fas-associated death domain
FDMA	Frequency division multiple access
GPx	Glutathione peroxidase
GSH	Reduced glutathione
GSM	Global system for mobile communications
GSSG	Glutathione disulphide
HF	High frequency
IARC	International Agency for Research on Cancer
ICNIRP	International Commission on Non-Ionizing Radiation Protection
iDEN	Integrated digital enhanced network
MOMP	Mitochondrial outer membrane potential
MX	3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone
ODC	Ornithine decarboxylase
PBMC	Peripheral blood mononuclear cells
RF	Radiofrequency
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RTL	Radial transmission line
SAR	Specific absorption rate
SEM	Standard error of mean
SOD	Superoxide dismutase
TDMA	Time division multiple access
TEM	Transverse electromagnetic
TPA	12-O-tetradecanoylphorbol-13-acetate
UV	Ultraviolet (radiation)
WLAN	Wireless local area network
WPC	Wire patch cell

LIST OF ORIGINAL PUBLICATIONS

Chapter 2	Anne Höytö, Ari-Pekka Sihvonen, Leena Alhonen, Jukka Juutilainen, Jonne Naarala. Modest increase in temperature affects ODC activity in L929 cells: Low-level radiofrequency radiation does not. Radiation and Environmental Biophysics 45(3): 231-235, 2006.
Chapter 3	Anne Höytö, Jukka Juutilainen, Jonne Naarala. Ornithine decarboxylase activity of L929 cells after exposure to continuous wave or 50 Hz modulated radiofrequency radiation - a replication study. Bioelectromagnetics 28(7): 501-508, 2007.
Chapter 4	Anne Höytö, Jukka Juutilainen, Jonne Naarala. Ornithine decarboxylase activity is affected in primary astrocytes but not in secondary cell lines exposed to 872 MHz RF radiation. International Journal of Radiation Biology 83(6): 367-374, 2007.
Chapter 5	Anne Höytö, Mikko Sokura, Jukka Juutilainen, Jonne Naarala. Radiofrequency radiation does not significantly affect ornithine decarboxylase activity, proliferation, or caspase-3 activity of fibroblasts in different physiological conditions. Submitted.
Chapter 6	Anne Höytö, Jukka Luukkonen, Jukka Juutilainen, Jonne Naarala. Proliferation, oxidative stress, and cell death in cells exposed to 872 MHz radiofrequency radiation and oxidants. Radiation Research, In press.

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Chapter 1.

General introduction to proliferation and cell death related effects of radiofrequency (RF) radiation on cell cultures

1 Literature review

1.1 Exposure to RF radiation

In August 2007 the number of mobile communication subscribers all over the world surpassed the 3 billion mark and the number continues to increase by 850 connections every minute (Wireless Intelligence 2007). This rapidly expanding use of mobile communication has aroused public concern about the possible health effects of exposure to the radiofrequency (RF) radiation utilised in cell phones. A number of studies have been carried out to investigate whether mobile communication devices are safe for their users, but the results have been somewhat conflicting. The studies performed include epidemiological, human, *in vivo*, as well as *in vitro* studies.

RF radiation is a form of electromagnetic radiation. Electromagnetic radiation is a self-propagating wave in space consisting of oscillating electric (E) and magnetic (H) fields. Electromagnetic radiation is classified into several different forms of radiation according to the frequency (f, number of oscillations per second) of the wave; generally RF radiation is defined as that covering the frequencies from 100 kHz to 300 GHz (Figure 1). RF radiation is further specified as microwaves, if the frequency of the radiation is between 300 MHz and 300 GHz.

Electromagnetic radiation is designated as non-ionising when the frequency is below 30 PHz and as ionising at higher frequencies. Thus, RF radiation belongs to the non-ionising radiation type. Photons of non-ionising radiation do not carry enough energy to ionise atoms or molecules. Instead of producing charged ions when passing through matter, non-ionising radiation induces electric fields and currents and can generate heat (visible light and UV radiation are also able to excite electrons). Thus, RF radiation is not anticipated as being able to interfere with DNA directly but it can evoke an increase of tissue temperature at high exposure levels. However, at the low power range utilised in mobile phones, this heating effect is minimal.

Mobile networks utilise different frequencies in different parts of the world. In Europe, GSM (Global system for mobile communications) networks are in 900 and 1800 MHz bands, whereas the DAMPS (digital advanced mobile phone system) networks in America utilise the 800 and 1900 MHz bands. In digital mobile communication systems, the RF signal is pulse modulated to

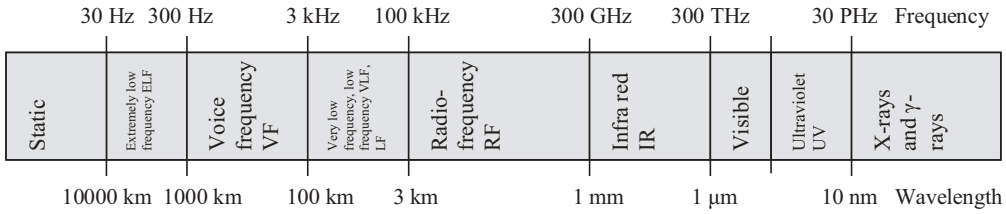

Figure 1. The electromagnetic spectrum. Wavelength decreases with increasing frequency.

carry information and the modulation frequency also varies depending on the system. Pulse modulation is a form of amplitude modulation, which applies pulsed modulation of the carrier wave by a low-frequency signal. Analogue mobile phones utilise frequency modulation, a process which involves modulation of the frequency around the basic frequency (Juutilainen & de Seze 1998). GSM mobile phones use pulse modulation at 217 Hz, with a pulse width of 577 μs, whereas DAMPS work at 50 Hz, with a pulse width of 6.67 ms (also referred to in the US as TDMA, time division multiple access; however, also GSM uses the TDMA method). It has been suggested that the effects of RF radiation on living organisms may depend on the modulation characteristics of the signal, although evidence of modulation-specific effects is weak (Juutilainen & de Seze 1998); therefore it is necessary to be aware of the modulation type.

Today, human exposure to RF radiation mainly comes from mobile phone handsets and antennae, but also other sources exist. For example, cordless phones, wireless local area networks (WLANs), electronic article surveillance systems and other anti-theft devices, induction cookers, computer monitors and television sets, as well as broadcast antennae for radio and television are sources of RF radiation (AGNIR 2003). Also some diagnostic appliances utilise RF fields, for example magnetic resonance imaging and tumour ablation devices. In occupational settings, exposure to RF radiation is common among the operators of dielectric heaters, induction heaters, diathermy machines, plasma discharge machines, and radars (AGNIR 2003).

To protect people from any possible adverse effects of RF radiation, the International Commission on Non-Ionizing Radiation Protection (ICNIRP) has established international guidelines on exposure levels (ICNIRP 1998). The maximum limit for human whole body exposure is 0.08 W/kg for the general public and 0.4 W/kg in occupational settings. For partial body exposure, the exposure limits are 2 W/kg and 4 W/kg for the general public and workers, respectively. The exposure limits are set as Specific Absorption Rates (SARs), which is a common measure for RF field exposure. SAR reflects the rate at which RF energy is absorbed into the body when exposed to a RF field and it depends on the characteristics of the field as well as the properties of the exposed body. In the EU, the SAR limit for mobile phones in the market is 2 W/kg, averaged over 10 g of tissue. In the US, the corresponding limit is 1.6 W/kg evaluated over a volume of 1 g of tissue.

To study the possible health effects of RF radiation at the frequencies used in mobile communication, different types of exposure systems have been developed. For example, transverse electromagnetic mode (TEM) cells, high frequency (HF) chambers, radial transmission lines (RTL), waveguides, and wire patch cells (WPC) have been commonly used for *in vitro* RF radiation exposures (Schönborn *et al.* 2001).

Exposure set-ups for *in vitro* studies must comply with the needs of the cultured cells, i.e. appropriate temperature, pH, and humidity. These can be achieved by placing the exposure unit inside an ordinary cell culture incubator or by providing the unit with equipment to maintain the environment required for cell culture. TEM cells (Figure 2) have been widely used in exposing cell cultures, as they are small, self-contained and they fit within normal cell culture incubators (Schönborn *et al.* 2001). The drawback is that the RF field is not very homogeneous with larger dish numbers.

In HF chambers, a large number of flasks can be exposed but the SAR distribution is

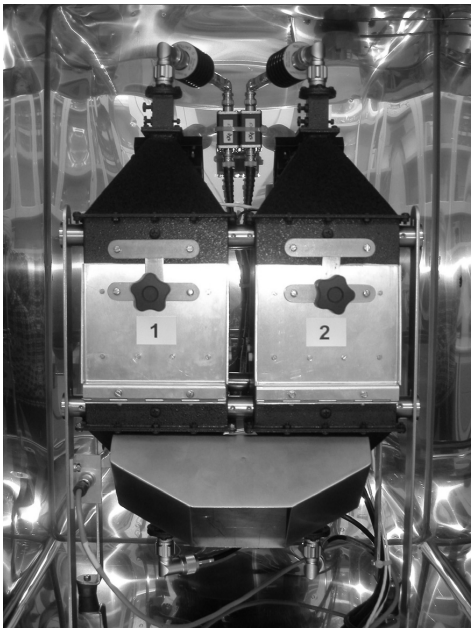

Figure 2. TEM cell housed in an incubator. This kind of set-up was used in one part of the present study.

non-homogeneous, the optimal cell culture environment is hard to support, and they are costly. The RTL exposure set-up allows exposure of several dishes in a rather homogenous field, but the system is not very efficient. With a waveguide exposure set-up, it is possible to achieve good homogeneity, but only in a few dishes (Figure 3). Wire patch cells are small enough to fit into incubators and they produce a rather homogeneous field. However, their characterisation is still incomplete.

Sometimes mere mobile phones have been used as the source of exposure in experimental studies. However, the use of specialised exposure set-ups does confer numerous advantages compared to exposing with a mobile phone, although all exposure set-ups suffer from their own limitations.

RF radiation exposure set-ups can be designed and characterised such that SAR level and distribution of the field in the subject are known. This is the fundamental requirement for a scientific study on the effects of RF radiation on health. Additionally, many set-ups allow testing of continuous wave signal and different modulation types over a range of exposure levels. Some set-ups even permit temperature compensation, therefore enabling exposure to higher SAR levels but still under non-thermal conditions. None of these requirements and advantages can be achieved if a mobile phone is used to expose cells, animals or humans by merely placing the phone next to the biological system being tested. If the exposure parameters are not perfectly known, replication of the study is not possible and reproducibility (which is a fundamental requirement in science) cannot be assessed.

The use of specialized exposure system leads to a number of problems. Namely, different temperature control methods used in exposure set-ups complicates comparison between

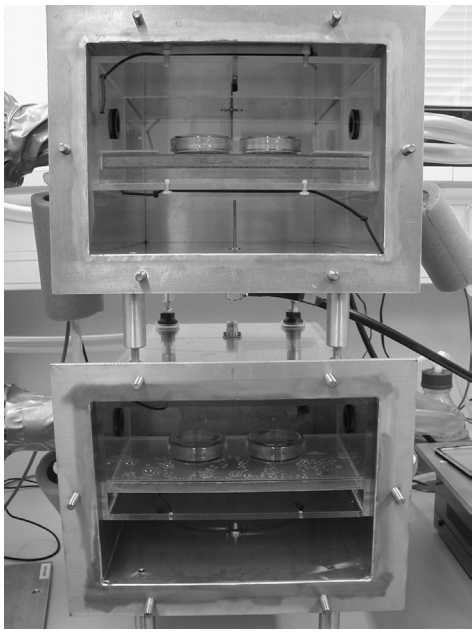

Figure 3. Waveguide exposure chamber equipped with water cooling. This kind of set-up was used in a part of the present study.

different equipment, as some have water cooling whereas the others use air cooling. Furthermore, many exposure systems have no temperature compensation at all. In some exposure set-ups chemical buffering of cell culture medium has to be used to maintain the pH at the appropriate level, whereas in some equipment, the CO_2 concentration of the atmosphere buffers the medium.

The dimensions of the exposure chamber also restrict the choice of cell culture vessels, together with the fact that usually SAR estimation has been performed only for one type of cell culture dishes or flasks. This may lead to difficulties in some types of analyses, since after exposure, the cells may need to be transferred into a different type of vessel, for example into multiwell plates.

1.2 Introduction to biological effects of RF radiation

A number of studies have been performed during recent years to investigate the human health effects of RF radiation. The studies have focused mainly on carcinogenicity, but also other end points have been extensively studied.

Studies with human subjects have focused on effects of low level RF exposure on brain function during consciousness and sleep mainly using electroencephalograms (EEG) and performance measures (Krewski *et al.* 2001a&b, Cook *et al.* 2002&2006). Virtually all of the studies that have measured EEG have reported some changes, but the significance of these findings remains unclear as the majority of performance assays have not indicated any changes in neuropsychological tasks of attention and working memory (Cook *et al.* 2002&2006, Russo *et al.* 2006, Wilén *et al.* 2006, Fritzer *et al.* 2007). However, significant changes in long time recall, trail making test, visual short-term memory, symbol digit modalities, and sustained attention to response test during or immediately after RF exposure have been reported (Lass *et al.* 2002, Lee *et al.* 2003, Smythe & Costall 2003, Keetley *et al.* 2006).

Epidemiological studies on exposure to mobile phone radiation and carcinogenicity have generally resulted in virtually no increased cancer risks but many of these studies have suffered from too short follow-up periods and poor exposure assessment (see Moulder *et al.* 2005). However, in recent years, a few reports have been published that have mostly overcome these limitations. These studies mostly belong to the large international collaboration study (Interphone). Generally these studies have detected no increased cancer risks (Berg *et al.* 2006, Takebayashi *et al.* 2006, Hours *et al.* 2007), but some studies have suggested that long term use of mobile phones (e.g. over ten years) may be causally related to some types of brain cancers, particularly glioma (Hepworth *et al.* 2006, Schüz *et al.* 2006) and acoustic neuroma (Lönn *et al.* 2004). In Sweden after conducting a series of case-control studies, the research group of Hardell claimed that an increase in acoustic neuroma risk is causally related to mobile phone use (Hardell *et al.* 1999, 2002a&b, 2003, 2005), but their studies have been criticised because of possible selection and information bias (Moulder *et al.* 2005).

In vivo studies have focused mainly on tumourigenesis and genotoxicity, but also other end points have been studied. In tumourigenicity studies, the carcinogenic potential of RF radiation alone has been evaluated as well as its potential to promote the development of tumours induced by known carcinogens (Heikkinen *et al.* 2001&2006, Heikkinen 2006). Tumour prone animal strains have also been used to evaluate

the carcinogenicity of RF radiation (Heikkinen *et al.* 2003). Tumourigenicity studies have generally been negative; only a few studies have found any evidence for a carcinogenic potential of RF radiation (see Moulder *et al.* 2005). The most notable positive study is that of Repacholi *et al.* (1997), who exposed Eμ-Pim1 transgenic mice to 900 MHz GSM-type RF radiation at average SAR levels of 0.13–1.4 W/kg for 18 months (2 x 30min/d, 7 d/wk) and reported a two-fold increase in the incidence of lymphoma. However, a replication study (Utteridge *et al.* 2002) and an extension study (Oberto *et al.* 2007) both failed to confirm the original result.

The genotoxicity of RF radiation has been studied by measuring single and double strand DNA breaks, induction of micronuclei, and DNA damage using the alkaline comet assay in brain, peripheral blood cells, bone marrow cells, and liver of rats, as well as peripheral blood cells, bone marrow cells, keratinocytes, and spleen lymphocytes of mice (Heynick *et al.* 2003, Trosic *et al.* 2002&2004, Görlitz *et al.* 2005, Verschaeve *et al.* 2006, Juutilainen *et al.* 2007). These studies have generally detected no significant effects on genotoxic endpoints. However, Lai and Singh (1995, 1996&1997) reported effects of RF radiation on single strand and double strand DNA breaks in rat brain and Trosic *et al.* (2002&2004) reported increased micronucleus frequencies in peripheral blood and bone marrow erythrocytes of rats exposed to RF radiation.

In addition to tumourigenicity and genotoxicity, other effects of RF radiation for example on behaviour, blood-brain barrier, melatonin levels, and ornithine decarboxylase (ODC) activity have been studied in animals (Krewski *et al.* 2001a&b). Some early behavioural studies claimed to observe effects on spatial memory task performance, but the replication studies failed to confirm the findings (Sienkiewicz *et al.* 2005). Interestingly, a recent study indicated that long-term RF radiation exposure of juvenile rats may even improve learning and memory (Kumlin *et al.* 2007). An increase in *in vivo* blood-brain barrier permeability has been reported by several authors (Hossmann and Hermann, 2003). However the increase may have resulted from elevated temperature or immobilization stress of the animals and the reported alterations were small and reversible. More recent studies have not found any significant effects of RF radiation on blood-brain barrier integrity (Finnie *et al.* 2001, 2002, 2004, 2006a&b). Studies with rats have reported increased ODC activity in brain tissue after long-term exposure to RF radiation (Paulraj *et al.* 1999, Paulraj & Behari 2002), but no effects after short-term exposure (Stagg *et al.* 2001). Mason *et al.* (2001) reported also no effects on ODC activity in a SENCAR mouse skin carcinogenesis model.

Studies on cell cultures have mainly focused on exploring the mechanisms of the possible biological effects of RF radiation, and the endpoints have included genomic effects, oxidative stress, effects on gene and protein expression, cell membranes, proliferation, transformation, and cell death. There has been much interest in the interaction of RF radiation with genetic material, with about 40 reports on this subject being published (for a review, see Vijayalaxmi & Obe 2004). The cytogenetic tests used have included chromosomal aberration, micronucleus, sister chromatid exchange, and aneuploidy assays and in some studies have also involved co-exposure with known genotoxic agents. A clear majority of the studies have indicated that RF radiation has no effects on genotoxic endpoints either alone or in combination with other factors (30 out of 45 investigations, Vijayalaxmi & Obe 2004). However, some reports have pointed towards the possibility

that RF radiation could induce genetic defects. For example, Diem *et al.* (2005) reported increased formation of DNA single and double strand breaks in transformed rat granulosa cells after exposure to RF radiation and Baohong *et al.* (2005) suggested that RF radiation may enhance the DNA damage induced by mitomycin C and 4-nitroquinoline-1-oxide in human lymphocytes. However, in their attempt to confirm the results of Diem *et al.* (2005), Speit and his co-workers (2007) failed to detect any effects of RF radiation on the incidence of micronuclei or DNA strand breaks.

Genomic effects of RF radiation at gene and protein expression level have been studied rather extensively in recent years. Although generally exposure to RF radiation has not resulted in changes in gene or protein expression, a number of studies have described altered gene expression after RF radiation exposure (for example Nylund & Leszczynski 2004, Lee *et al*. 2005, Buttiglione *et al*. 2007, Zhao *et al*. 2007a&b). Upregulation in apoptosis-related genes (Lee *et al*. 2005, Buttiglione *et al*. 2007, Zhao *et al*. 2007b), downregulation in cell cycle genes (Lee *et al*. 2005), and effects on the cytoskeleton, signal transduction pathway, and metabolism associated genes (Zhao *et al*. 2007a), as well as cytoskeleton-related protein expression (Nylund & Leszczynski 2004) have been reported.

It has been suggested that cell membranes are a target for RF radiation in cells. RF radiation has been reported to affect the properties of a variety of ion channels in membranes (reviewed by Repacholi 1998). Decreased rates of channel protein formation, decreased frequency of single channel openings, and increased rates of rapid, burst-like firing have been described, but the findings have been equivocal and their relevance to human health is unknown.

Transformation of normal cells into an altered growth state after RF radiation exposure has been studied by several investigators, e.g. Balcer-Kubiczek and Harrison (1985, 1989 & 1991) have reported increased transformation in C3H/10T1/2 mouse embryo fibroblasts either after RF radiation exposure alone or in combination with X-rays, benzo[a]pyrene, or 12-O-tetradecanoylphorbol-13-acetate (TPA). However, Cain *et al.* (1997) and Roti-Roti *et al.* (2001) were unable to detect any effects on cellular transformation after RF radiation exposure of the same cell line with or without X-ray and TPA treatments. In a recent study, BALB/3T3 cells were exposed to RF radiation alone or in combination with 3-methylcholanthrene and TPA and, again, no effects on transformation were detected (Hirose *et al.* 2007).

Several biological endpoints related to growth, oxidative stress and cell death have been extensively studied in cell cultures exposed to RF radiation and will be described in detail in the following pages.

1.3 ODC activity and proliferation

Ornithine decarboxylase (EC 4.1.1.17) is the first and rate limiting enzyme in the pathway of polyamine biosynthesis (Figure 4). The polyamines, putrescine, spermidine, and spermine, are involved in the control of cell replication, differentiation, tumour initiation, and tumour promotion (Thomas and Thomas, 2001). An increase in cellular ODC activity has been reported to be involved in carcinogenesis (O'Brien *et al.* 1982; Auvinen *et al.* 1992), as several tumour promoters, for example phorbol esters such as TPA, induce ODC activity and a high level of ODC activity has been found in a number of premalignant conditions. In addition, several known oncogenes such as c-myc, c-fos, and H-ras increase ODC activity apparently by enhancing transcription of the ODC gene (Bello-Fernandez *et al*. 1993, Hurta *et al.* 1993, Wrighton & Busslinger 1993).

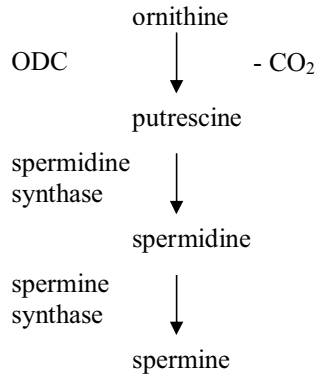

Figure 4. Polyamine biosynthesis pathway.

Overexpression of ODC by itself is not sufficient to induce tumours in normal cells, but an increase in cellular ODC activity can enhance tumour development in initiated premalignant cells (Clifford *et al.* 1995). Furthermore, cells overexpressing ODC exhibit an increased ability to achieve anchorage-independent growth (Kubota *et al.* 1997).

RF radiation has been reported to increase ODC activity in cultured cells (Byus *et al.* 1988; Litovitz *et al.* 1993&1997; Penafiel *et al.* 1997). However, known tumour promoters tend to induce the ODC activity in cell cultures up to 20–30 fold (O'Brien and Diamond 1977; Azadniv *et al.* 1995), which is much higher than the 1.4–1.9-fold changes reported after RF radiation (Byus *et al.* 1988; Litovitz *et al.* 1993&1997; Penafiel *et al.* 1997). Nonetheless, one can speculate that increased ODC activity after RF radiation exposure may serve as an indicator of the biological interaction between RF energy and living cells. In several studies, the reported increase in ODC activity was associated with amplitude-modulated RF radiation. Thus, no effects were seen with non-modulated, i.e., continuous wave (CW) radiation at similar exposure levels (Litovitz *et al.* 1993&1997, Penafiel *et al.* 1997). These kinds of modulation-specific effects would be particularly relevant to mobile phone exposure, as amplitude-modulated signals are used in digital mobile phone systems.

Byus and his co-workers (1988) exposed Reuber H35 hepatoma, Chinese hamster ovary (CHO), and human 294 T melanoma cells in a TEM cell to 450 MHz RF fields sinusoidally amplitude modulated at 16 Hz, at a power density of 1.0 mW/cm^2 (peak envelope power) for 1 h and observed a 50 % increase in ODC activity. Also the enhancement of ODC activity induced by addition of TPA (1.6 μM) was further stimulated by RF radiation exposure in H35 and CHO cells. These workers established a window of amplitude modulation between 10 and 20 Hz that was able to increase the ODC activity.

The results of Litovitz *et al.* (1993) indicated that a 915 MHz microwave field amplitude modulated at 55, 60, or 65 Hz at SAR level of 2.5 W/kg for 8 h increased ODC activity by 90 % in murine L929 fibroblast cells exposed in TEM cells. In contrast, a continuous wave signal did not produce any effects on cellular ODC activity. From the same laboratory, Penafiel *et al.* (1997) and Litovitz *et al.* (1997) reported that a 40 % increase in ODC activity in L929 cells was detected after exposure to TDMA digital cellular telephone signal (840 MHz, burst modulated at 50 Hz, with 33 % duty cycle, SAR 2.5 W/kg). Furthermore, signals with an amplitude modulation at 60 Hz and pulsed amplitude modulation at 50 Hz did induce ODC activity, whereas a signal modulated with speech, the signal of an analogue cellular phone, or a signal frequency modulated at 60 Hz did not affect ODC activity. Various exposure times between 2 and 24 hours were used with the effect being most pronounced after exposure for 8 h.

However, in an attempt to replicate the study of Penafiel *et al.* (1997), Desta *et al.* (2003) failed to observe any increase in ODC activity in L929 cells after 8 h of exposure to 835 MHz RF radiation with the modulation typical to TDMA mobile phones at SAR levels from < 1 W/kg to 15 W/kg. On the contrary, Desta and his co-workers reported a decrease in cellular ODC activity at SAR levels above 6 W/kg producing a temperature increase of more than 1 °C in the cell culture medium. Under these conditions, ODC activity was decreased by 25 % after exposures at 6–10 W/kg.

Some earlier studies have also reported that hyperthermia decreases cellular ODC activity both *in vitro* and *in vivo*. In those studies, however, the temperatures were considerably higher (40–42 °C) than those relevant to RF radiation exposure studies and were at levels that can induce a heat shock response. A 99 % decrease of ODC activity was reported in CHO cells kept at 42.4 °C for 3 h compared to control cells maintained at 37 °C (Gerner *et al.* 1983). The same investigators also exposed the cells to slow heating from 37.0 to 42.4 °C over 3 h, and reported that ODC activity decreased during heating. Verma and Zibell (1985) incubated pieces of skin from adult mice at 40 °C, and noted 66 % and 93 % decline in ODC activity at 1 h and 5 h of incubation, respectively, compared to skin pieces kept incubated at 37 °C. Mice were also exposed to whole-body hyperthermia (rectal temperature 41 °C) for 1 h. The ODC activity of kidneys decreased by 78 %, compared to control mice kept at room temperature (rectal temperature 38.4 °C).

The proliferation of cells in normal tissue is strictly controlled. Increased proliferation of cells, for example due to induced ODC activity, may lead to uncontrolled growth of transformed cells and this can cause the development of cancer (Clifford *et al.* 1995). A number of studies have addressed the effects of RF radiation on cellular proliferation but the results have varied depending on cell type and exposure conditions. Cleary *et al.* (1990a&b, 1996) reported increased proliferation in human LN71 glioma cells, human mononuclear lymphocytes and cytolytic T lymphocytes (CTLL-2) stimulated with interleukin 2. In these experiments, cells were exposed for 2 h to 27 or 2450 MHz CW or pulse modulated (50 Hz) signals at SAR levels up to 50 W/kg under isothermal conditions. Exposures at 27 MHz were carried out in a coaxial line RF exposure chamber and a waveguide exposure chamber was used to achieve exposures at 2450 MHz. An increased proliferation rate was also detected in transformed human epithelial amnion cells (AMA) cultured at 35 or 39 °C and exposed in a TEM cell to a 960 MHz signal modulated at 217 Hz at a SAR level of 2.1 mW/kg (Velizarov *et al.* 1999).

Four studies have resulted in opposite conclusions. A decreased proliferation rate was measured in CTLL-2 cells exposed for 2 h to 2450 MHz CW radiation in a waveguide exposure chamber at SAR levels of 25 and 50 W/kg (Cleary *et al.* 1996). AMA cells exposed in a TEM cell for 20–40 min to a 960 MHz signal modulated at 217 Hz at SAR levels of 0.021–2.1 mW/kg showed a decreased proliferation rate (Kwee & Raskmark 1998). Capri and her co-workers (2004b) exposed human peripheral blood mononuclear cells (PBMC) stimulated with a mitogen (phytohemagglutinin) to a 900 MHz GSM modulated RF field inside a TEM cell at a SAR level of 76 mW/kg for 1 h/day for 3 days with a reduced proliferation rate being observed after 72 h in culture. Takashima *et al.* (2006) exposed CHO cells and human malignant glioma MO54 cells to 2.45 GHz CW RF radiation at a wide range of SAR values (0.05–200 W/kg) either continuously or intermittently (1 s on/2–29 s off) for 2 h. Continuous exposure to the highest SAR level (200 W/kg) decreased the proliferation rates

in both cell lines, and the authors concluded, that the outcome was due to increased temperature of the cell culture medium (44.1 °C).

Several authors have reported that RF radiation had no significant effects on cellular proliferation of different cell types. Krause *et al.* (1991) exposed murine L929 cells to 2.45 GHz CW radiation at a SAR of 130 mW/g for 4 h and Stagg *et al.* (1997) exposed rat C6 glioma cells and primary fetal rat brain cultures to 836.55 MHz TDMA modulated signal at 0.59–59 μW/g but observed no effects on proliferation measured as doubling rate of the cells. Exposure of human blood lymphocytes to CW 2450 MHz RF radiation at a SAR level of 12.46 W/kg for 90 min had no effects on incidence of binucleated cells as a measure of proliferation (Vijayalaxmi *et al.* 1997). Mouse embryonic stem cell-derived neural progenitor cells were exposed to 1.71 GHz GSM (217 Hz) modulated signal at 1.5 W/kg intermittently (5 min on/30 min off) for 48 h and the number of cells in 1st, 2nd, and 3rd mitosis were not affected (Nikolova *et al.* 2005). Lixia *et al.* (2006) performed exposures at 1.8 GHz with GSM modulation at SAR levels of 1–3 W/kg for 2 h with human lens epithelial cell line SRA01/04. They reported no effects on bromodeoxyuridine incorporation in RF radiation exposed cells. Merola *et al.* (2006) reported no effects on the metabolic conversion of a tetrazolium salt, WST-1, in human LAN-5 neuroblastoma cells exposed to GSM modulated 900 MHz RF signal at 1 W/kg for 24–72 h. Sanchez and her co-workers (2006) exposed human reconstructed epidermis to 900 MHz GSM signal for 48 h, using a SAR level of 2 W/kg and noted no change in the numbers of activated nuclei. Human peripheral lymphocytes did not show any alterations in cytokinesis-block proliferation indices after exposure at SAR levels of 1–10 W/kg to 900 MHz RF signal with GSM modulation for 24 h (Scarfi *et al.* 2006).

1.4 Oxidative stress

Oxidative stress is an imbalance between the formation of free radicals (reactive oxygen or nitrogen species) and the ability of antioxidant systems to remove these reactive molecules in biological organisms (Valko *et al*. 2007). Oxidative stress may cause damage to proteins, lipids, and DNA and thus impair their normal action. Oxidative stress has been implicated in a number of human diseases, for example cancer, diabetes mellitus, ischemia/reperfusion injury, inflammatory diseases, neurodegenerative disorders, as well as having a role in the ageing process (Valko *et al.* 2007). The most important reactive oxygen species (ROS) are the oxygen molecule (dioxygen) itself, superoxide anion $O_2^{\bullet -}$, hydroxyl radical $^{\bullet}OH$, and peroxyl radicals $ROO^{\bullet}$. In addition, hydrogen peroxide H_2O_2 is an important oxidising agent in cells. Similarly, nitric oxide $NO^{\bullet}$ and peroxynitrite anion $ONOO^-$ are the major reactive nitrogen species (RNS) and overproduction of RNS leads to nitrosative stress. However, ROS and RNS are vital to normal functioning of cells, as they are involved for example as a defence against infectious agents and in biological signalling (Valko *et al*. 2006).

Organisms have developed a variety of defence mechanisms to prevent damage due to oxidative stress (Cadenas 1997). The defence mechanisms against free radical-induced oxidative stress involve preventative mechanisms, repair mechanisms, physical defences, and antioxidant defences. Several proteins, e.g. superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) are enzymatic antioxidants. Non-enzymatic antioxidants include ascorbic acid (Vitamin C), α-tocopherol (Vitamin E), glutathione (GSH), carotenoids, and

flavonoids. GSH is the major thiol antioxidant and redox buffer in the cell and the oxidised form of GSH is glutathione disulphide (GSSG).

Generally, cell proliferation is stimulated in a more reducing environment (maintained by elevated levels of glutathione and thioredoxin) whereas a slight shift towards a mildly oxidising environment initiates cell differentiation. A further shift towards a more oxidising environment in the cell can trigger either apoptosis (programmed cell death) or necrosis. While apoptosis is evoked by moderate oxidising stimuli, necrosis is induced by intense oxidising effects. When applied to carcinogenesis, slight to moderate oxidative stress can act as a tumour promoter, moderate to severe oxidative stress is mutagenic, and a high level of oxidative stress induces apoptosis or necrosis (Voehringer 1999, Schafer & Buettner 2001).

Induction of oxidative stress has been reported after exposure to a variety of factors. For example, certain chemicals, ionising radiation (X- and γ-rays), and UV radiation can all cause increased ROS/RNS production. Chemicals that may evoke oxidative stress include for example chlorinated compounds, barbiturates, metal ions, and quinines (Valko *et al.* 2006).

A recent study proposed that oxidative stress may be the underlying mechanism responsible for the reported effects of RF radiation on cells (Friedman *et al.* 2007). The authors suggested that NADH oxidase in plasma membrane could generate ROS to stimulate matrix metalloproteinases after RF radiation exposure. However, the exposure of Rat1 and HeLa cells was carried out in an incubator equipped with an emitting antenna and the distribution and SAR level of the RF field in the cell plates was not reported. The authors only stated that the field intensity was 0.07 mW/cm^2 and the frequency was 875 MHz. Furthermore, cellular ROS production was not measured directly, instead enhanced NADH oxidation activity was concluded to trigger ROS production. One study has shown an increase in cellular ROS production after coexposure to RF radiation and iron ions in rat lymphocytes (Zmyslony *et al.* 2004). The cells were exposed to 930 MHz CW RF radiation at a SAR level of 1.5 W/kg in a TEM cell. After 5 and 15 min coexposures to $FeCl_2$ (10 μg/ml) and the RF field, the intracellular ROS production measured using a fluorescent probe 2',7'-dichlorofluorescein-diacetate (DCF-DA) was elevated by 16.6 % and 14.6 %, respectively. Exposure to RF radiation alone did not significantly affect ROS production.

Other studies have not been able to demonstrate effects in different oxidative stress parameters in various cell lines (Hook *et al.* 2004a, Lantow *et al.* 2006a&b, Simko *et al.* 2006, Zeni *et al.* 2007). Mouse J774.16 macrophages were exposed to 835.62 MHz CW and 847.74 MHz code division multiple access (CDMA) modulated signals at 0.8 W/kg in RTL exposure system for 20-22 h with γ-interferon and bacterial lipopolysaccharide being used to stimulate the cells (Hook *et al.* 2004a). The oxidant levels, antioxidant levels, oxidative damage, and nitric oxide production were assayed but no effects were detected in any of those parameters. Lantow and coworkers (2006a&b) exposed primary human monocytes, human Mono Mac 6, and K562 cells to 1800 MHz CW and GSM modulated signals at SAR levels of 0.5–2 W/kg for 45 min. The GSM signal included various modulations: discontinuous transmission mode (DTX), non-DTX, Talk, with some exposures being carried out intermittently (5 min on/5 min off). The exposures were carried out in single-mode resonator cavities with coexposure to 1 μM TPA also being used. No significant effects after RF radiation exposure on cellular ROS release (measured

as reduction of dihydrorhodamine 123 to rhodamine) and superoxide anion radicals (measured as reduction of nitro blue tetrazolium to formazan) were measured either with or without TPA. Simko *et al.* (2006) also exposed human Mono Mac 6 cells under similar conditions as Lantow *et al.* (2006a&b), but the coexposures were carried out with ultrafine particles (< 0.1 μm). RF radiation exposure was not able to further enhance the superoxide anion radical production induced by ultrafine particles. Finally, Zeni and her coworkers (2007) exposed murine L929 fibroblasts to CW or GSM modulated 900 MHz RF radiation in a waveguide exposure set-up at SAR levels of 0.3 and 1.0 W/kg. The cells were exposed for 10 or 30 min and co-exposure with 500 μM 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone (MX) was used in some of the exposures. No effects on cellular ROS production (determined using DCF-DA) were measured after RF radiation exposure either alone or in combination with MX.

1.5 Cell death

Cell death can be divided into programmed cell death (apoptosis) and necrosis, but recently, in addition to necrosis, also other non-apoptotic cell death forms have been recognised, such as autophagic cell death, mitotic cell death, and caspases-independent cell death (Blank & Shiloh 2007). Apoptosis can be defined as the type of cell death that is accompanied by rounding-up of the cell, retraction of pseudopodes, reduction of cellular volume (pyknosis), condensation of the chromatin, fragmentation of the nucleus (karyorhexis), little or no ultrastructural modification of cytoplasmic organelles, plasma membrane blebbing, and maintenance of an intact plasma membrane until late stages of the process, all of which ultimately leads to formation of membrane surrounded vesicles called apoptotic bodies (Kroemer *et al.* 2005). Necrosis features cytoplasmic swelling, mechanical rupture of the plasma membrane, dilation of cytoplasmic organelles (mitochondria, endoplasmic reticulum and Golgi apparatus), as well as moderate chromatin condensation (Kroemer *et al.* 2005). Necrosis but not apoptosis causes inflammation in tissues.

Apoptosis is executed by a family of zymogenic proteases known as caspases (cysteinyl aspartate-specific proteinases) that dismantle the cell in an orderly fashion by cleaving an array of intracellular substrates. Two major apoptotic pathways have been described in eukaryotic cells: the extrinsic and intrinsic pathways (Blank & Shiloh 2007). The extrinsic signalling pathway involves the binding of extracellular ligands (e.g., FasL) to cell surface receptors (e.g., Fas/CD95), resulting in the recruitment of cytosolic adaptor proteins such as FADD (Fas-associated death domain), activation of initiator caspases (e.g., caspase-8), and subsequent activation of the downstream effector caspases (caspase-3, -6, and -7) (Nagata 1999). The latter may also be achieved in the extrinsic pathway through induction of mitochondrial outer membrane permeabilisation (MOMP), with subsequent formation of the apoptosome (cytochrome c/Apaf-1/procaspase-9 complex) (Schafer & Kornbluth 2006). The intrinsic (or mitochondrial) pathway is triggered by various intracellular stimuli (for example DNA damage, cytoskeletal damage, endoplasmic reticulum stress, and macromolecular synthesis inhibition) that induce MOMP, which is followed by release of cytochrome c and the formation of the apoptosome (Spierings *et al.* 2005). Usually caspase-9 and -3 are activated to execute apoptosis.

In recent years, the effect of exposure to RF radiation on apoptosis has been investigated by many researchers. Although the majority

of studies have indicated no effects on cellular apoptosis, some reports have hinted at increased apoptosis after RF radiation exposure. Peinnequin *et al.* (2000) found that proliferation after Fas-induced apoptosis was decreased in human Jurkat T-cells after exposure to 2.45 GHz CW radiation at a SAR level of 4 W/kg for 48 h. The RF field was produced by a custom made horn antenna inside an incubator and the control cells were kept in the same incubator at a SAR level of < 0.08 W/kg. Maeda *et al.* (2004) reported induction of apoptosis in human LoVo cells after pulsed (2 x 3 s/day for 3 days) microwave treatment. However, they did not provide details of the characteristics of the signal and SAR levels used in the experiments. Also activation of both p53-dependent and -independent apoptosis pathways have been reported in human T-lymphoblastoid leukaemia cells exposed in a TEM cell to unmodulated 900 MHz signal at a SAR level of 3.5 mW/kg for 2–48 h (Marinelli *et al.* 2004). Markkanen and his co-workers (2004) reported that apoptosis measured by annexin V-FITC staining was enhanced in mutant yeast cells exposed in a waveguide chamber for 1 h to GSM modulated 872 and 900 MHz radiation at SAR levels of 3.0 W/kg and 0.4 W/kg, respectively, in coexposure with UV radiation. CW RF radiation did not affect apoptosis. Caraglia *et al.* (2005) proposed that RF induced apoptosis was mediated through the inactivation of the ras/Erk survival signalling due to enhanced degradation of ras and Raf-1 in human epidermoid cancer cells, which were exposed to a 1.95 GHz CW signal at a SAR level of 3.6 W/kg in a waveguide chamber for 1–3 h. Primary cultures of murine neurons and astrocytes have undergone upregulation of apoptosis genes after RF radiation exposure for 2 h (Zhao *et al.* 2007b). However, these investigators used a mobile phone (1900 MHz) placed on top of a Petri dish containing the cells and therefore the actual SAR level and distribution of RF radiation are not known.

Port *et al.* (2003) did not detect any effects on the number of apoptotic cells after exposure to a 0.4 GHz field with a pulse repetition frequency of 1 Hz for 6 min. The exposure level (field strength about 50 kV/m) was reported to be about 25 times higher than the ICNIRP reference levels for occupational exposure but not sufficient to cause any significant effects due to increased temperature. Human myeloid leukaemia cells (HL-60) were used in the experiments and the exposures were carried out in a TEM cell. Capri and her co-workers (2004a&b) exposed human peripheral blood mononuclear cells in either a waveguide chamber (1800 MHz) or a TEM cell (900 MHz) to RF radiation alone or together with the apoptosis-inducing agent 2-deoxy-D-ribose. The 1800 MHz field was modulated with three different GSM modulation schemes, GSM basic, GSM DTX, and GSM Talk, and the 900 MHz signal was either unmodulated or GSM modulated. No differences in apoptosis as measured by annexin V-FITC staining between sham-exposed and RF exposed cells were detected, irrespective of whether or not the cells were treated with an apoptosis inducing agent. Also Hook *et al.* (2004b) reported that the annexin V affinity assay did not detect any signs of apoptosis in a human lymphoblastoid leukaemia cell line (Molt-4). They exposed the cells in a RTL irradiator to 847.74 MHz CDMA, 835.62 MHz frequency-division multiple-access (FDMA), 813.56 MHz integrated digital enhanced network (iDEN), and 836.55 MHz TDMA signals for up to 24 h. The SAR levels were 3.2 W/kg for CDMA and FDMA, 2.4 or 24 mW/kg for iDEN, and 2.6 or 26 mW/kg for TDMA. Belyaev and his co-authors (2005) reported that exposure in a TEM cell to a GSM modulated 915 MHz field did not change the number of morphologically apoptotic cells or the fragmentation of DNA in human peripheral blood lymphocytes. The cells were derived

from hypersensitive and healthy persons and the exposures were carried out in a TEM cell at a SAR level of 37 mW/kg for 2 h. Nikolova *et al.* (2005) exposed embryonic stem cell-derived neural progenitor cells to 1.71 GHz GSM modulated signal at 1.5 W/kg for 48 h (intermittently 5 min on/30 min off). They reported no changes in the mitochondrial membrane potential or in the fluorescent activated cell sorting (FACS) analysis of nuclear apoptosis after the exposure.

Gurisk *et al.* (2006) exposed human neuroblastoma SK-N-SH cells to a GSM modulated 900 MHz signal at a SAR level of 0.2 W/kg in a TEM cell for 2 h with apoptosis being assessed by propidium iodide/YO-PRO-1 dyeing. No differences were detected between sham-exposed and exposed samples. Hirose and co-workers (2006) reported no effects on annexin V-FITC affinity assay or apoptosis related gene expression analysis in human glioblastoma A172 and human IMR-90 fibroblasts after 24–48 hours of RF exposure. The exposures were carried out in a beam-shaped RF exposure incubator employing a horn antenna producing RF fields at 2.1425 GHz with or without W-CDMA modulation at SAR levels of 80–800 mW/kg. Joubert *et al.* (2006&2007) studied the effects of CW and GSM modulated 900 MHz radiation on apoptosis in human neuroblastoma SH-SY5Y cells and rat primary cortical neurons. Exposure levels were 0.25 W/kg (GSM) and 2 W/kg (CW) and a wire patch cell was used for 24 h exposures. No effects on cellular apoptosis after RF radiation exposure were detected with three different techniques i.e. 4',6-diamino-2-phenylindole (DAPI) staining, flow cytometry with double staining (TUNEL and PI), or measurement of caspase-3 activity by fluorometry.

Lantow *et al.* (2006c) reported no effects on annexin V/FITC affinity in human Mono Mac 6 cells after exposure to a 1800 MHz GSM-DTX signal alone or in combination with TPA or gliotoxin. The 12 h exposures were carried out in single-mode resonator cavities at SAR level of 2.0 W/kg. A coexposure approach was also used by Merola *et al.* (2006) to study the effects of 900 MHz radiation with GSM modulation (SAR 1 W/kg) on human neuroblastoma LAN-5 cells. Camptothecin was used to induce apoptosis and the duration of RF radiation exposure was 24 or 48 h. No evidence of cellular apoptosis was observed in the caspase activation assay after exposure to RF radiation. Sanchez and her co-workers (2006) exposed normal human epidermal keratinocytes and fibroblasts in a WPC exposure set-up to a 900 MHz signal modulated at 217 Hz (GSM) at a SAR level of 2 W/kg for 48 h. No alteration in apoptosis was detected in the annexin V/FITC affinity assay. In another study, Sanchez *et al.* (2007) exposed the same cells in a waveguide set-up to a 1800 MHz signal modulated with 217 Hz at the same SAR level (2 W/kg) for 2 h and this type of signal also did not affect cellular annexin V/FITC affinity. Recently, Chauhan *et al.* (2007) reported that the incidence of apoptosis was not affected in human TK6, HL-60, or Mono Mac 6 cells after exposure to RF radiation. They exposed cells to pulse-modulated 1.9 GHz field at SAR levels of 1 and 10 W/kg for 6 h in cylindrical waveguides with apoptosis being assessed by neutral comet assay.

The viability of cells has not been found to be affected by RF radiation (Hook *et al.* 2004a, Gurisk *et al.* 2006). Viability was measured as the ability of cells to exclude trypan blue dye. There were no differences between exposed and sham-exposed cells in either of the studies. Hook *et al.* (2004a) studied how mouse J774.16 macrophages responded to exposure to either 835.62 MHz CW or 847.74 MHz CDMA modulated RF field at 0.8 W/kg. Gurisk *et al.* (2006) exposed human neuronal SK-N-SH and monocytic U937 cells to a 900

MHz signal with GSM modulation at 217 Hz (SAR 0.2 W/kg).

1.6 Summary of *in vitro* studies related to growth, oxidative stress and cell death

One common feature of the *in vitro* studies on growth, oxidative stress, and cell death after exposure to RF radiation has been that the results have been equivocal. Some studies have shown increased ODC activity in cells exposed to modulated RF radiation (Byus *et al*. 1988, Litovitz *et al.* 1993&1997, Penafiel *et al.* 1997) but one replication study was unable to discover any RF field related effects (Desta *et al.* 2003), except for a heating-related decline in cellular ODC activity at high SAR levels.

The results of the proliferation assays have varied from inhibition to stimulation with many studies finding no effects at all. The effects on proliferation reported after exposures at high SAR levels may be related to the elevated temperature. If there are true effects of weak, nonthermal RF radiation on cell proliferation, these effects do seem to be cell type specific and furthermore characteristics of the employed RF signal may affect the outcome. However, modulation-specific responses have not been reported, as both CW and modulated signals have affected the proliferation of exposed cells.

The level of cellular oxidative stress has generally not been affected after RF radiation exposure. However, there are two studies reporting that RF radiation induced effects might be mediated by alterations in cellular ROS production (Zmyslony *et al.* 2004, Friedman *et al.* 2007).

Although the majority of studies regarding cellular apoptosis have been negative, increased cellular apoptosis after exposure to RF radiation has been reported in several papers. Effects have been reported from both CW and modulated signals, but one study reported that the effects were only evident with the modulated signal (Markkanen *et al.* 2004). RF radiation has not been reported to affect cellular viability in any studies.

2 Aims of the present study

The aim of the present study was to assess proliferation, cell death, and oxidative stress related effects of mobile phone radiation using cell cultures. The more specific aims were as follows:

- To replicate studies reporting effects on ODC from exposure to DAMPS modulated RF radiation (modulation frequency 50 Hz).
- To perform ODC activity studies with GSM modulated RF radiation in an exposure system with improved temperature control and a more homogenous distribution of the RF field.
- To compare the effects of CW and modulated RF radiation.
- To study the effects of RF radiation on cell proliferation, oxidative stress, and cell death.
- To compare the responses of different cell types to RF radiation.
- To test if combined exposure with agents inducing cellular stress could enhance the possibility of detecting weak effects of RF radiation.

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Chapter 2.

Modest increase in temperature affects ODC activity in L929 cells: low-level radiofrequency radiation does not

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1 Abstract

The effects of low-level radiofrequency (RF) radiation and elevated temperature on ornithine decarboxylase (ODC) activity were investigated in murine L929 fibroblasts. The cells were exposed at 900 MHz either to a pulse-modulated (pulse frequency 217 Hz; GSM-type modulation) or a continuous wave signal at specific absorption rate (SAR) levels of 0.2 W kg^{-1} (0.1–0.3 W kg^{-1}) and 0.4 W kg^{-1} (0.3–0.5 W kg^{-1}) for 2, 8, or 24 h. RF radiation did not affect cellular ODC activity. However, a slight increase in temperature (0.8–0.9°C) in the exposure system lead to decreased ODC activity in cell cultures. This was verified by tests in which cells were exposed to different temperatures in incubators. The results show that ODC activity is sensitive to small temperature differences in cell cultures. Hence, a precise temperature control in cellular ODC activity studies is needed.

2 Introduction

Ornithine decarboxylase (ODC) is the first and rate-limiting enzyme in the biosynthesis of polyamines. Polyamines are essential for cell growth and differentiation, and their role in cell proliferation and carcinogenesis has been extensively studied [1]. ODC activity is stimulated, for example, by phorbol ester tumour promoting agents, and it has been reported that radiofrequency (RF) radiation may affect it. Two groups have reported that exposure to RF radiation modulated in various ways may induce cellular ODC activity maximally by 90% [2–5]. Desta et al. [6] failed to show any effects of TDMA modulated RF signal on cellular ODC activity. In some of these previous studies [2–4], continuous wave (CW) microwave exposure also was tested, but this type of exposure did not induce any changes in cellular ODC activity. Effects of RF fields with modulation parameters similar to the GSM mobile communication system have not been studied for effects on ODC activity.

In this study, we investigated possible effects of low-level, 900 MHz RF fields on ODC activity in L929 cells. Both GSM-type pulse-modulated signal and continuous wave exposure were tested. The exposures were carried out using a newly designed waveguide exposure chamber that has a temperature control system for the cell culture dishes. Furthermore, effects of increased temperature (<1°C) on cellular ODC activity were studied.

3 Materials and methods

3.1 Cell culture

L929 murine fibroblasts were purchased from ECACC (European Collection of Cell Cultures, Salisbury, UK). These cells are of the same origin as the cells from American type culture collection (ATCC), which Litovitz and co-workers [3, 5] used in their studies. The cells were grown at 37°C in 5% CO_2 in plastic 75 cm^2 flasks (Nunc, Roskilde, Denmark) in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal calf serum, penicillin (50 U ml^{-1}), and streptomycin (50 µg ml^{-1}) (all obtained from Gibco, Invitrogen, Carlsbad, CA, USA). Cells were harvested by trypsinisation. Approximately 20 h prior to exposure, cells were seeded into four 55 mm diameter glass Petri dishes (Schott, Mainz, Germany). For shorter (2 and 8 h) exposures, 2 x 10^6 cells per dish, and for 24 h exposures, 1 x 10^6 cells per dish were plated in 5 ml of culture medium to provide sub-confluent cultures at the beginning of the exposure. Cells were evenly distributed in the dishes and two randomly selected dishes were placed in the exposure chamber and the other two in the sham-exposure chamber. To avoid excess activation of ODC, medium was never changed prior to the exposure.

3.2 RF exposure

The design of the waveguide exposure system aimed at good control of the temperature (± 0.3°C) of the cell culture vessels and provision of a uniform specific absorption rate (SAR) distribution. The exposure chamber was an aluminium RF-resonator with plastic cultivation chamber and a water circulation heat exchanger. A control unit adjusted the temperature of the water, which circulated under a glass surface on which the cell-culture dishes were placed. Glass Petri dishes were used instead of plastic dishes. The plastic dishes have a plastic rim around their bottom while glass dishes are flat, thus ensuring better thermal contact between the cell culture medium and the circulating water. There were two identical chambers, one for exposure and the other for a simultaneous sham-exposure.

The system allowed lowering the circulating water temperature to compensate for cell-culture heating produced by absorbed RF power at high SAR levels (≥ 1.5 W kg^{-1}). A temperature adjustment curve was determined, which showed the water temperature setting for each SAR level and aimed at keeping the measured temperature of the cell culture medium constant (± 0.3°C) at SAR values up to 6 W kg^{-1}. CO_2 (5%) and warm air (approximately 37°C) were fed into the cultivation chambers from a cell culture incubator (Jouan IG 150, Saint-Herblain, France).

The RF power source consisted of a signal generator model SMY 02 (Rohde & Schwartz, Munich, Germany), a 217 Hz GSM modulator (STUK, Finland), and R720FC power amplifier (RF Power Labs, USA). RF power was fed into the exposure chamber with a monopole post, and the electric field inside the chamber, below the heat exchanger, was monitored from another monopole post with a NAS 828.6017.02 RF-power meter (Rohde & Schwarz, Munich, Germany).

Specific absorption rate estimation was based on FDTD calculations completed using commercial code XFDTD® (Remcom Corporation, State College, PA, USA). The numerically calculated SAR distribution within the cultivation dish was verified. The first approach was to measure the temperature rise with a miniature Vitek-type thermistor temperature probe (BSD Medical, Salt Lake City, USA) [7]. However, the electric field coupled to the probe, making the results unreliable. Positioning the probe perpendicular or parallel to the electric field produced ten fold difference to the measured SAR

values. Second, the simulations were compared to the measured electric field profile inside the exposure chamber. When the calculated and measured field values were scaled to the dissipated power within the chamber, the agreement between the field values was excellent (maximum 5% difference). The calculated SAR within a Petri dish was 2.5 W kg^{-1} ± 30% for 1 W of dissipated power.

Cells were exposed for 2, 8, or 24 h to 900 MHz RF fields, using either pulse modulation at 217 Hz (modulation characteristics typical to the GSM mobile phone system) or continuous wave exposure, at SAR levels of 0.2 W kg^{-1} (0.1–0.3 W kg^{-1}) and 0.4 W kg^{-1} (0.3–0.5 W kg^{-1}). These SARs were selected to study effects of RF radiation levels to which mobile phone users are actually exposed. The exposure times were similar to those used by Penafiel et al. [5]. During exposure, cells were kept at 37°C and 5% CO_2. No temperature compensation was needed during these low-level exposures. Two dishes were exposed simultaneously, and two dishes were sham-exposed. This was repeated 3–5 times at each exposure level and duration (n = 6–10).

3.3 Assessment of small temperature differences

To determine if (1) there were performance differences between the temperature regulation systems of the exposure and sham chambers and (2) if such temperature differences affected ODC activity, cell culture dishes were placed in both chambers. RF was not applied in the exposure chamber, producing what could be called a sham–sham comparison. Cell culture dishes were incubated in exposure chambers for 2, 8, or 24 h with cell densities equal to those used in real exposures. The temperature of the culture medium in the dishes was also measured with a Fluke 52 k/j thermometer (Fluke Corporation, Everett, WA, USA).

In separate additional experiments cells were incubated in ordinary cell culture incubators (HeraCell, Heraeus Instruments GmbH & Co KG, Hanau, Germany) at different temperatures for 2 h to determine the effect of small temperature differences on ODC activity. In one incubator, cells were exposed to 37.6°C and in the other to 36.5°C. Temperatures of the culture medium were measured with the same Fluke 52 k/j thermometer and thermocouple mentioned earlier. Magnetic fields of the incubators were measured (Holaday HI-3624 ELF magnetic field meter with Holaday ELF magnetic field sensor p/n 491017, Eden Prairie, MN, USA), and a 1.1 μT 50 Hz field was detected in both incubators at the position of the cell dishes. Cells were handled exactly in the same way as in the exposures in the waveguide system, e.g. 2 x 10^6 cells were plated on glass Petri dishes for exposures.

3.4 ODC assay

After exposure, the medium was removed and the cells were washed twice with ice-cold (4°C) Ca^{2+}- and Mg^{2+}-free PBS. Then 1 ml of ice-cold PBS was added to each dish, and the cells were detached by scraping. The cell suspension was centrifuged (500 x g, 5 min, 4°C) and the supernatant discarded. Dry cell pellets were frozen in -70°C for later analysis of ODC activity.

ODC activity of coded samples was assayed in a blinded manner according to the method described by Jänne and Williams-Ashman [8]. Units of ODC activity were expressed as pmol $^{14}CO_2$ generated per h per mg protein at 37°C. Due to day-to-day variation of ODC activity in control cultures results are expressed also as ODC activity ratio ± SEM (n=3–5), which is calculated by dividing the mean ODC activity of the two simultaneously exposed cultures by the mean of the two non-exposed control cultures. Changing of culture medium was

used as a positive control and after 4 h exposure to fresh medium, mean ODC activity in control cultures was 571 ± 122 pmol h^{-1} mg^{-1} compared to 13,194 ± 1,129 pmol h^{-1} mg^{-1} in cultures exposed to fresh medium. Since exposure to magnetic field has been shown to induce ODC activity in L929 cells [9], exposure to 50 Hz sine wave magnetic field of 10 μT for 4 h was used as an additional positive control. After exposure, mean ODC activity was 3,357 ± 528 pmol h^{-1} mg^{-1} in controls and 2,285 ± 246 pmol h^{-1} mg^{-1} in exposed cells. Thus, instead of inducing ODC activity, magnetic field exposure decreased ODC activity by 28.5%.

3.5 Statistical analyses

The data of RF exposure studies were analysed with a four-way analysis of variance (ANOVA) for ODC activity levels of exposed and control cells. The four factors were "Treatment" (exposed and sham-exposed), "Modulation" (GSM-modulated and continuous wave signals), "Exposure level" (0.2 and 0.4 W kg^{-1}), and "Time" (2, 8, and 24 h). Analysis was made using SPSS (SPSS Science, Chicago, IL, USA).

The data of temperature tests in incubators were analysed with two-tailed, paired *t* test using GraphPad Prism (GraphPad Software Inc, San Diego, CA, USA). Mean of two dishes exposed and the mean of two dishes simultaneously sham-exposed was considered as a pair. A two-tailed test was selected because it was not possible to predict direction of differences.

A *P* value lower than 0.05 was considered statistically significant in both analyses.

4 Results

The results of the initial analysis (Table 1, columns 1–5) may be interpreted to suggest that low-level RF radiation decreases ODC-activity in exposed cells. In four-way ANOVA test, the factor "Treatment" (exposed and sham-exposed) was found statistically significant (P = 0.002), as well as "Time" (2, 8, and 24 h, P < 0.001) and interaction of these two (P = 0.03) (coefficient of determination $R2$ = 0.745). However, the sham–sham experiments (both chambers without RF power) showed that there was a difference in ODC activity between the chambers. The ODC-activity was consistently lower in the "exposure" chamber (without any RF radiation) than in the sham-exposure chamber after 2 and 24 h exposures. Therefore, in Table 1, columns 7 and 8, the results were corrected by normalization factors for each time point derived from the sham-sham testing of the system (Table 2). For example, the ODC activity ratio was 0.719 at 2 h in sham–sham exposures; hence the ODC activities at 2 h of RF exposure were divided by this factor.

The normalized results (Table 1) show that there is no difference between the ODC activities of the RF-exposed and the sham-exposed cells (factor "Treatment", P= 0.584). Factor "Time" is still significant with a P < 0.001, reflecting the change of ODC activity during the growth phase of the cell cultures ($R2$ = 0.759). The reason for the difference in ODC activity between the two chambers was found in additional measurements: the temperature in water circulation heat exchanger in the exposure chamber (37.5 ± 0.3°C) was, on average, 0.8°C higher than in the sham exposure chamber (36.7 ± 0.3°C), despite the temperature control.

Tests with standard cell culture incubators (Table 3) confirmed the importance of small temperature differences: ODC activity was 43 ± 5.1% ($P \leq 0.01$, paired *t*-test) lower in cells grown at 37.6°C compared to cells grown at 36.5°C.

Table 1. Results of the sham-sham comparison. The mean C (sham exposure chamber) and mean E (exposure chamber without any RF radiation) values are expressed as pmol $^{14}CO_2$ generated/60 min/mg protein at 37°C ± SEM of the n control (C) and n exposed (E) cultures. ODC activity ratio is calculated by dividing the mean ODC activity of the two simultaneously exposed cultures by the mean of the two non-exposed control cultures. ODC activity ratio is expressed as mean ± SEM.

Exp time (hrs)	n	Mean C	Mean E	ODC activity ratio
2	8	3686.7 ± 169.0	2685.2 ± 285.5	0.719 ± 0.06
8	6	1983.8 ± 480.0	1996.7 ± 472.5	1.002 ± 0.05
24	6	1480.1 ± 373.9	1224.9 ± 359.5	0.820 ± 0.10

5 Discussion

These results indicate that neither GSM-modulated nor continuous wave 900 MHz RF radiation, at SARs of 0.2 or 0.4 W kg^{-1}, affect ODC activity in L929 cells. The effects of higher SARs and other modulations, such as those used by Litovitz et al. [3] and Byus et al. [2] remain to be investigated. The present data confirm the results of the sensitivity of ODC activity to heating [6]. In our studies, ODC activity of L929 cells was found even more susceptible to heating than in this previous study. In incubators, less than 1°C increase in temperature decreased ODC activity by 43%. Desta and his group reported statistically significant decrease in ODC activity only after heating of over 1.5°C. The sensitivity of ODC activity to small temperature differences is an interesting finding that is important for any further studies of RF field effects on ODC.

Some earlier studies have also reported that hyperthermia decreases cellular ODC activity both in vitro and in vivo. In those studies, however, temperatures were considerably higher (40–42°C) than those used in the present study and were at levels that induce the heat-shock response. Verma and Zibell [10] incubated pieces of skin from adult mice at 40°C, which resulted in an ODC activity decrease of 66% at 1 h, and a 93% decrease at 5 h of incubation, compared to skin pieces incubated at 37°C. In the same study, mice also were exposed to whole-body hyperthermia (rectal temperature 41°C) for 1 h. The ODC activity of kidneys decreased by 78%, compared to control mice kept at room temperature (rectal temperature 38.4°C). Gerner et al. [11] reported a 99% decrease of ODC activity in CHO cells kept at 42.4°C for 3 h compared to control cells kept at 37°C. They also exposed the cells to a slow heating from 37.0 to 42.4°C in 3 h, and ODC activity was reported to decrease during heating.

The present results suggest that temperature-related ODC changes are likely to have occurred in previous in vitro RF field bioeffects studies, if no adequate methods were used to control small temperature differences between exposed and control cultures. For example, Litovitz et al. [3] reported ODC activity changes at a SAR of 2.5 W kg^{-1}. In our exposure system, exposure to SARs of 1.5 W kg^{-1} and 2.5 W kg^{-1} increased temperature of cell cultures by 0.33 and 0.56°C, respectively, if no temperature compensation was used. However, the previous studies [2, 5] were able to detect differences in cellular ODC activities between various modulation frequencies

Table 2. Results of the exposures with 0.2 and 0.4 W/kg, GSM modulated (217 Hz) or continuous wave (CW) RF radiation. Column headings for columns identical to Table 1 are as established in Table 1. Additional columns: Normalization factor derived from Table 1 is used to normalize ODC activities of exposed samples due to temperature differences in exposure and sham-exposure chambers. Normalized mean E is the mean of E values divided by the normalization factor. Normalized ODC activity ratio is calculated as ODC activity ratio, but using normalized E values.

Exp time (hrs)	n	Mean C	Mean E	ODC activity ratio	Norm factor	Norm mean E	Norm ODC activity ratio
0.2 W/kg GSM							
2	6	3058.3 ± 417.6	2298.6 ± 302.0	0.77	0.719	3196.3 ± 419.9	1.07
8	6	1426.1 ± 204.1	1253.3 ± 121.9	0.90	1.002	1250.7 ± 121.6	0.90
24	8	1655.4 ± 108.9	1437.9 ± 198.4	0.88	0.820	1754.2 ± 242.0	1.07
0.2 W/kg CW							
2	6	3222.8 ± 408.1	2292.6 ± 331.0	0.72	0.719	3187.9 ± 460.3	1.00
8	10	1390.9 ± 147.4	1148.9 ± 122.6	0.87	1.002	1146.4 ± 122.4	0.87
24	8	885.7 ± 90.7	752.4 ± 109.4	0.84	0.820	917.9 ± 133.5	1.02
0.4 W/kg GSM							
2	6	3949.0 ± 532.3	2616.9 ± 430.0	0.66	0.719	3638.8 ± 597.9	0.92
8	6	1786.3 ± 381.5	1532.5 ± 266.5	0.84	1.002	1529.2 ± 265.9	0.84
24	6	907.9 ± 4188.7	892.1 ± 132.9	1.06	0.820	1088.3 ± 162.2	1.29
0.4 W/kg CW							
2	6	3869.5 ± 648.8	2685.1 ± 305.2	0.72	0.719	3733.6 ± 424.3	1.01
8	6	1517.7 ± 67.4	1289.2 ± 176.3	0.85	1.002	1286.4 ± 175.9	0.84
24	6	865.3 ± 196.0	707.4 ± 234.8	0.70	0.820	863.0 ± 286.4	0.85

Table 3. Results of the 2 h temperature tests in incubators. Mean is the average of the ODC activity expressed as pmol $^{14}CO_2$ generated/60 min/mg protein at 37°C ± SEM of the n samples exposed to either 36.5 or 37.6°C. ODC activity ratio is as established in Tables 1 and 2. The *p* value is the probability that observed differences between paired control and exposed samples are due to a chance.

Exp temperature (°C)	n	Mean	ODC activity ratio	*p*
36.5	6	7405.7 ± 640.4		
37.6	6	4298.4 ± 607.3	0.57 ± 0.05	0.01

(different from the modulation used by the present study) under the same SAR level, which indicates the possibility of other than temperature-related effects. On the other hand, Desta et al. [6] reported no other than temperature-related effects on ODC activity after 8 h exposure to SARs < 1–15 W kg^{-1} in an attempt to repeat the Penafiel et al. [5] study. The present results show that cellular ODC activity is remarkably sensitive to small temperature differences that are within the range of normal cell culture conditions. This indicates that very precise temperature control is essential in studies on cellular ODC activity.

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Chapter 3.

ODC activity of L929 cells after exposure to continuous wave or 50 Hz modulated radiofrequency radiation - A replication study

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1 Abstract

A replication study with some extensions was made to confirm enhancement of ornithine decarboxylase (ODC) activity in murine L929 fibroblasts after radiofrequency (RF) field exposure reported in earlier studies. L929 cells purchased from two cell banks were exposed for 2, 8, or 24 h to continuous wave or DAMPS (burst modulated at 50 Hz, with 33% duty cycle) signals at specific absorption rate (SAR) levels of 2.5 or 6.0 W/kg. Exposures were carried out in Crawford and waveguide chambers, at frequencies 835 and 872 MHz, respectively. The results did not confirm findings of previous studies reporting increased ODC activity in RF-exposed cells. When Crawford cell exposure system was used, ODC activity was either not affected (in the case of 8 or 24 h exposures) or decreased after 2 h exposure at the highest SAR level (6 W/kg). The decrease was most pronounced when cooling with air flow was not used, and is most likely related to increased temperature. The minor methodological differences (use of antibiotics, increased sensitivity of ODC assay) are not likely to explain the inconsistency of the findings of the present and previous studies. Different results were obtained in experiments with the waveguide system that involves more efficient temperature control. In this exposure system, ODC activity was increased after 8 h exposure at 6 W/kg. Further studies are warranted to explore whether this finding reflects a true non-thermal effect. The present study did not provide evidence for modulation-specific effects reported in earlier studies.

2 Introduction

Ornithine decarboxylase (ODC) (EC 4.1.1.17) is the first and rate limiting enzyme in the pathway of polyamine synthesis. Polyamines putrescine, spermidine, and spermine are involved in the control of cell replication, differentiation, tumor initiation, and tumor promotion [Thomas and Thomas, 2001]. Several tumor promoters induce ODC activity and radiofrequency (RF) radiation has also been reported to increase ODC activity in cultured cells [Byus et al., 1988; Litovitz et al., 1993, 1997; Penafiel et al., 1997]. Byus et al. [1988] reported that ODC activity was increased by 50% in Reuber H35 hepatoma, Chinese hamster ovary, and human 294T melanoma cells after 1 h exposure to a 450 MHz microwave field, sinusoidally amplitude modulated at 16 Hz (peak-envelope-power 1.0 mW/cm^2). Studies of Litovitz et al. [1993] indicated that microwave field (915 MHz) amplitude modulated at 55, 60, or 65 Hz at specific absorption rate (SAR) level of 2.5 W/kg for 8 h increased ODC activity

of murine L929 fibroblast cells by 90%. Penafiel et al. [1997] and Litovitz et al. [1997] reported that a 40% increase in ODC activity in L929 cells was detected after 8 h of exposure to a time division multiple access (TDMA) digital cellular telephone signal (840 MHz, burst modulated at 50 Hz, with 33% duty cycle, SAR 2.5 W/kg).

The World Health Organization (WHO) has listed specific areas of research on molecular and cellular interactions of low-level RF fields, and ODC activity was included in the priority list [Repacholi, 1998]. Therefore, an attempt was made in the present study to replicate results of Penafiel et al. [1997].

The protocol described by Penafiel et al. [1997] was followed with minor modifications. As an exposure setup, a Crawford cell was used. Exposures were carried out with continuous and TDMA modulated 835 MHz RF fields at SAR levels of 2.5 and 6.0 W/kg for 2, 8, and 24 h. The higher exposure level was tested because Nikoloski et al. [2005] found that in the Crawford cell exposure system the SAR was 6.0 W/kg at 1 W input power, rather than 2.5 W/kg as reported by Litovitz et al. [1993]. However, these measurements were not necessarily an accurate reflection of Litovitz' measurements and therefore both exposure levels were included in this study. Additionally, exposures were carried out in a waveguide exposure setup with water cooling that allows efficient control against temperature increase resulting from RF field exposure. The same cell line (murine L929 fibroblasts) was used and cells were purchased from two cell banks to find out if there were differences between the cells obtained from different origins.

3 Materials and methods

3.1 RF exposure

RF exposures were carried out in a Crawford chamber and in a waveguide exposure chamber. The Crawford chamber setup was designed, optimized, manufactured and characterized by Foundation for Research on Information Technologies in Society (IT'IS, Zürich, Switzerland) and the description of dosimetry was published elsewhere [Nikoloski et al., 2005]. Compared to that used by Penafiel et al. [1997], a few modifications were made to improve the setup. The doors of the transverse electromagnetic mode (TEM) cells (model IFI CC110, Instruments for Industry, Inc., Ronkonkoma, NY) were enlarged and inside TEM cells shelves were added to assist placing of the cell culture flasks, power sensors were fitted to follow SAR level continuously, and ventilators were introduced to provide additional cooling of the cell cultures. These improvements increased the accuracy and reproducibility of exposures at desired SAR level. The temperature increase in cell culture medium was assessed to be 0.046 and 0.19 °C/(W/kg) with and without air cooling, respectively, and the system allowed us to compare ODC activities of cell cultures exposed to RF radiation with or without cooling. In our laboratory, the TEM cells were housed in a water jacketed Sanyo CO_2 incubator (Model MCO-175, Gunma, Japan). Exposures were carried out with 835 MHz continuous wave (CW) or DAMPS (Digital Advanced Mobile Phone Service) modulated signal with pulse repetition rate of 50 Hz and duty cycle of 1:3. SAR levels were 2.5 or 6.0 W/kg and the air flow was on to cool the exposed cells, except for a series of experiments performed without air cooling at 6.0 W/kg. Cell samples were exposed for 0, 2, 8, or 24 h and the exposure level, temperature of the air inside the TEM cells, and the performance of the air flow fans was continuously monitored by a computer

interface throughout the whole exposure. The setup enabled performing of experiments in a blinded manner.

The waveguide exposure system was designed and characterized in STUK (Radiation and Nuclear Safety Authority, Helsinki, Finland), and a description of the system was published earlier [Höytö et al., 2006]. Briefly, the waveguide exposure system provides a uniform SAR distribution (± 35%) in cell cultures and a water cooling system enables exposure in controlled temperature (± 0.3 °C). Modulation similar to that used in the DAMPS system (pulse repetition rate of 50 Hz, duty cycle of 1:3) was generated using an arbitrary waveform generator (model 75, Wavetek, San Diego, CA). Exposures were carried out with an 872 MHz signal at SAR level of 6.0 W/kg for 0 and 8 h.

3.2 Cell culture

L929 murine fibroblasts were purchased from ECACC (No. 98I020, European Collection of Cell Cultures, Salisbury, UK) and ATCC (No. 1532960, American Type Culture Collection). The cells were grown at 37 °C in 5% CO_2 in plastic 75 cm^2 flasks (Nunc, Roskilde, Denmark) in Eagle minimum essential medium (EMEM) (Gibco, Paisley, UK), supplemented with 5% heat-inactivated fetal calf serum (Gibco, Paisley, UK), penicillin (50 U/ml), and streptomycin (50 mg/ml) (Gibco, Paisley, UK), and 2 mM L-glutamine (Gibco, Paisley, UK). Cells were harvested by trypsinisation (trypsin, Gibco, Invitrogen Canada Inc., Burlington, Ont., Canada). Approximately 20 h prior to exposure in the Crawford chamber, cells from both origins were seeded into four plastic 25 cm^2 flasks (TPP, Techno Plastic Products, Trasadingen, Switzerland). Cells were plated at a density of 3 x 10^6 cells/flask in 5.0 ml of EMEM supplemented with 10 mM HEPES (Gibco, Paisley, UK) to obtain subconfluent cultures at the beginning of the exposure. Cells were incubated for 20 h at 37 °C in 5% CO_2 and just before exposure flasks were sealed. Cells were evenly distributed in the flasks and two randomly selected flasks/cell origin were placed in one exposure chamber and the other two flasks/cell origin in the other exposure chamber. For exposures in the waveguide chamber, 2 x 10^6 cells/dish in 5.0 ml of EMEM were plated in four 55 mm diameter glass Petri dishes (Schott, Mainz, Germany). Cells were evenly distributed in the dishes and two randomly selected dished were placed in the exposure chamber and the other two in the sham-exposure chamber. To avoid excess activation of ODC, medium was never changed prior to the exposure.

3.3 ODC assay

After exposure, the medium was removed and the cells were washed twice with ice-cold (4 °C) Ca^{2+}- and Mg^{2+}-free PBS. Then 1 ml of ice-cold PBS was added to each flask/dish, and the cells were detached by scraping. The cell suspension was centrifuged (500 *g*, 5 min, 4 °C) and the supernatant was discarded. Dry cell pellets were frozen in -70 °C for later analysis of ODC activity.

ODC activity of coded samples was assayed in a blinded manner according to the method described by Jänne and Williams-Ashman [1971] with some modifications. Briefly, frozen cell samples were lysed in buffer containing 25 mM Tris pH 7.4, 0.1 mM EDTA, 0.1% Triton X-100, and 1 mM DTT and vortexed. Homogenate was centrifuged (16060 *g*, 20 min, 4 °C) and an aliquot of supernatant was incubated using a shaking water bath for 1 hour at 37 °C in reaction mixture containing 100 mM Tris pH 7.4, 4 mM EDTA, 4 mM DTT, 0.2 mM L-ornithine, 0.2 mCi/assay ^{14}C-L-ornithine (specific activity 52.0 mCi/mmol), and 0.4 mM PLP. Addition of 2 M citric acid liberated $^{14}CO_2$ and samples were further incubated for 15 min. Liberated $^{14}CO_2$ was trapped in pleated filter paper wetted with Solvable

(Packard Instruments, Groningen, Netherlands). Filter papers were transferred to scintillation vials containing 3 ml OptiPhase HiSafe 3 scintillant (PerkinElmer Life Sciences, Fisher Chemicals, Loughborough, England) and the activity was measured using a liquid scintillation counter (Wallac WinSpectral 1414, PerkinElmer Life Sciences, Wallac Oy, Turku, Finland). Protein content of the samples was measured using Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA).

Units of ODC activity were expressed as pmol $^{14}CO_2$ generated/h/mg protein at 37 °C ± SEM (n = 6–14). Due to day-to-day variation of ODC activity in control cultures results are expressed also as ODC activity ratio ± SEM (n = 3–7), which is calculated by dividing the mean ODC activity of the two simultaneously exposed cultures by the mean of the two non-exposed control cultures.

3.4 Sham-sham experiments and positive controls

In sham–sham experiments, L929 cell flasks/dishes were placed in both exposure chambers and no RF field was applied to check that there are no such differences between the chambers that would cause differences in ODC activity. The cells were incubated for 2, 8 or 24 h in the Crawford cell system and for 8 h in the waveguide chamber.

In positive control experiments, medium change was used to induce and serum deprivation to suppress ODC activity. In these experiments, cells were incubated in normal medium (two dishes) or medium without serum (two dishes) for 20 h. After incubation, fresh medium was changed to one dish/each group and cells were further incubated for 4 h. Cells were frozen for later ODC activity assay.

3.5 Statistical analysis

Statistical analysis utilizing raw ODC values was performed using a linear mixed model analysis. In the model, replicate (each experimental run consisted of two exposed and two sham cultures) was included as a random factor and condition (exposed or sham) as a fixed factor. Each treatment (modulation, SAR level, exposure duration) was tested separately. To correct for multiple comparisons, corrected *P*-values were calculated as $1-(1-P)^N$, where N is the number of comparisons. Different exposure conditions (different cell lines, durations of exposure or exposure chambers) were considered separately, so N was never higher than 6. A *P*-value of 0.05 was used as the limit for statistical significance. The analyses were performed using SPSS for Windows release 11.5.1 (SPSS, Inc., Chicago, IL).

4 Results

Positive control experiments were carried out to demonstrate functioning of the ODC assay and responsiveness of L929 cells from different origins (ATCC and ECACC). Medium change did increase ODC activity (ECACC: 1390.8 pmol $^{14}CO_2$ generated/h/mg protein, ATCC: 1287.3 pmol $^{14}CO_2$ generated/h/mg protein) compared to control cultures (ECACC: 161.6 pmol $^{14}CO_2$ generated/h/mg protein, ATCC: 224.4 pmol $^{14}CO_2$ generated/h/mg protein). Serum deprivation had the opposite effect (ECACC: 129.8 pmol $^{14}CO_2$ generated/h/mg protein, ATCC: 103.2 pmol $^{14}CO_2$ generated/h/mg protein). Medium change after 20 h of serum deprivation induced ODC activity very strongly (ECACC: 3662.6 pmol $^{14}CO_2$ generated/h/mg protein, ATCC: 3331.6 pmol $^{14}CO_2$ generated/h/mg protein).

Sham–sham experiments were carried out to demonstrate similarity of the two

Table 1. ODC activities of L929 cells of ATCC origin after sham-sham experiments or various exposures in Crawford chamber with airflow on, exceptions indicated with symbols. n (C/E) = number of control (C) and exposed (E) samples. Mean C = mean and estimated mean ODC activity of control cultures as pmol $^{14}CO_2$ generated/h/mg protein ± SEM (estimated SEM). Mean E = mean and estimated mean ODC activity of exposed cultures as pmol $^{14}CO_2$ generated/h/mg protein ± SEM (estimated SEM). In sham-sham experiments C refers to chamber 1 and E to chamber 2 of Crawford chamber set-up. F = F statistic. (*p*) = uncorrected *p* value, *p* = *p* value corrected for multiple comparisons; significant *p* values (≤ 0.05) are indicated with bold font. ODC activity ratio = mean ODC activity of the two simultaneously exposed cultures divided by the mean of the two non-exposed control cultures.

Exp time h	Exp level W/kg	Modulation	n (C/E)	Mean C	Mean E	F	(*p*) *p*	ODC activity ratio
2	sham-sham		6/6	499.8 ± 58.3 (73.2)	499.7 ± 45.4 (73.2)	0.00	(0.998) 1.000	1.02 ± 0.08
	2.5	CW	10/10	290.5 ± 39.0 (55.2)	319.4 ± 37.3 (55.2)	2.32	(0.150) 0.623	1.13 ± 0.08
		DAMPS	10/10	244.6 ± 41.2 (65.1)	257.3 ± 47.6 (65.1)	0.44	(0.517) 0.987	1.03 ± 0.07
	6.0	CW	10/10	494.0 ± 83.2 (104.6)	374.3 ± 60.1 (104.6)	9.93	(**0.007**) **0.041**	0.80 ± 0.10
		DAMPS	14/14	417.6 ± 36.1 (43.5)	346.9 ± 29.3 (43.5)	6.44	(**0.020**) 0.114	0.89 ± 0.12
		DAMPS #	10/9	421.3 ± 66.7 (80.8)	282.2 ± 48.5 (81.3)	16.14	(**0.001**) **0.006**	0.67 ± 0.06
8	sham-sham		6/6	207.3 ± 19.4 (32.9)	226.1 ± 26.5 (32.9)	0.99	(0.350) 0.925	1.09 ± 0.06
	2.5	CW	10/10	164.6 ± 45.2 (68.1)	168.7 ± 46.5 (68.1)	0,13	(0.724) 0.999	1.05 ± 0.08
		DAMPS	10/10	248.2 ± 27.9 (36.9)	233.6 ± 24.8 (36.9)	0.67	(0.427) 0.965	0.95 ± 0.08
	6.0	CW	10/10	232.4 ± 33.4 (40.5)	248.2 ± 28.2 (40.5)	0.31	(0.589) 0.995	1.08 ± 0.10
		DAMPS	10/10	275.4 ± 40.1 (57.2)	239.6 ± 38.7 (57.2)	3.75	(0.073) 0.365	0.85 ± 0.07
		DAMPS #	10/10	177.9 ± 20.1 (32.6)	165.0 ± 25.9 (32.6)	0.69	(0.420) 0.962	0.89 ± 0.07
24	sham-sham		6/6	105.2 ± 19.9 (27.0)	98.0 ± 15.7 (27.0)	0.50	(0.502) 0.985	0.96 ± 0.07
	2.5	CW	10/10	77.2 ± 10.6 (17.2)	95.1 ± 14.0 (17.2)	4.02	(0.065) 0.332	1.27 ± 0.08
		DAMPS	10/10	71.5 ± 10.4 (18.4)	70.1 ± 15.1 (18.4)	0.03	(0.860) 0.999	0.92 ± 0.15
	6.0	CW	12/12	97.6 ± 23.0 (38.0)	99.1 ± 28.6 (38.0)	0.04	(0.855) 0.999	0.87 ± 0.11
		DAMPS	10/10	53.7 ± 8.8 (11.7)	57.5 ± 8.1 (11.7)	0.38	(0.546) 0.991	1.12 ± 0.10
		DAMPS #	10/10	28.3 ± 4.5 (6.1)	25.1 ± 4.2 (6.1)	1.12	(0.308) 0.890	0.94 ± 0.17

CW = continuous wave; DAMPS = burst modulated at 50 Hz, with 33% duty cycle; # Crawford chamber, no airflow

Table 2. ODC activities of L929 cells of ECACC origin after various exposures in Crawford chamber with airflow on, exceptions indicated with symbols. Column headings and abbreviations are as indicated in Table 1.

Exp time h	Exp level W/kg	Modulation	n (C/E)	Mean C	Mean E	F	(p) p	ODC activity ratio
2	sham-sham		6/6	521.6 ± 117.3 (78.9)	549.9 ± 32.5 (78.9)	0.18	(0.684) 0.999	1.17 ± 0.27
	2.5	CW	10/10	308.7 ± 78.4 (77.0)	327.4 ± 56.2 (77.0)	0.15	(0.708) 0.999	1.21 ± 0.31
		DAMPS	10/10	189.2 ± 32.1 (37.7)	162.0 ± 33.4 (37.7)	0.88	(0.365) 0.934	0.87 ± 0.16
	6.0	CW	10/10	311.5 ± 82.6 (79.1)	284.5 ± 53.3 (79.1)	0.48	(0.501) 0.985	1.08 ± 0.24
		DAMPS	12/12	256.5 ± 44.8 (65.6)	312.8 ± 63.2 (65.6)	1.16	(0.296) 0.878	1.26 ± 0.25
		DAMPS #	10/10	399.3 ± 126.1 (109.6)	278.4 ± 70.7 (109.6)	4.67	(**0.049**) 0.260	0.70 ± 0.08
8	sham-sham		6/6	157.5 ± 9.8 (27.4)	262.2 ± 34.2 (27.4)	7.28	(**0.022**) 0.125	1.68 ± 0.29
	2.5	CW	12/12	124.4 ± 37.3 (29.4)	89.2 ± 14.5 (29.4)	5.36	(**0.033**) 0.182	0.89 ± 0.19
		DAMPS	10/10	220.2 ± 29.0 (22.8)	193.0 ± 21.4 (22.8)	0.71	(0.410) 0.958	0.99 ± 0.23
	6.0	CW	10/10	167.7 ± 26.0 (22.9)	145.3 ± 16.6 (22.9)	1.65	(0.219) 0.773	0.90 ± 0.11
		DAMPS	10/10	327.0 ± 110.2 (102.3)	276.2 ± 64.7 (102.3)	3.51	(0.082) 0.402	0.83 ± 0.08
		DAMPS #	10/10	409.4 ± 124.3 (100.1)	341.8 ± 66.0 (100.1)	0.79	(0.390) 0.948	0.91 ± 0.21
24	sham-sham		6/6	115.3 ± 36.8 (35.8)	134.3 ± 23.4 (35.8)	2.40	(0.160) 0.649	1.27 ± 0.17
	2.5	CW	10/10	120.8 ± 61.7 (48.9)	108.6 ± 25.0 (48.9)	0.25	(0.625) 0.997	1.26 ± 0.25
		DAMPS	10/10	127.5 ± 14.5 (15.1)	113.8 ± 11.5 (15.1)	0.41	(0.528) 0.989	0.95 ± 0.13
	6.0	CW	10/10	99.2 ± 32.7 (37.6)	103.6 ± 31.9 (37.6)	0.04	(0.850) 0.999	0.99 ± 0.13
		DAMPS	10/10	87.0 ± 25.2 (19.7)	90.2 ± 11.2 (19.7)	0.08	(0.781) 0.999	1.38 ± 0.34
		DAMPS #	12/12	94.3 ± 8.4 (10.4)	96.1 ± 11.6 (10.4)	0.02	(0.890) 0.999	1.07 ± 0.20

CW = continuous wave; DAMPS = burst modulated at 50 Hz, with 33% duty cycle; # no airflow

chambers used for RF exposure and sham exposure. There were no significant differences between the two exposure chambers in either the Crawford or the waveguide setups (Tables 1–3).

L929 cells (ATCC and ECACC) were exposed in a Crawford chamber or a waveguide chamber to radiofrequency radiation at two SAR levels (2.5 and 6.0 W/kg) for 2, 8, or 24 h. In the Crawford chamber with air cooling, expected heating of the cell culture medium was 0.12 °C for exposures at SAR level of 2.5 W/kg and 0.28 °C for exposures at SAR level of 6.0 W/kg. Without air cooling, the expected temperature rise was 1.14 °C for exposure at 6.0 W/kg. In the waveguide exposure chamber, water cooling maintained medium temperature constant (± 0.3 °C).

In the Crawford chamber without airflow, a 33 % decrease in ODC activity was measured in ATCC cells after 2 h of exposure to DAMPS modulated RF radiation at 6.0 W/kg (Table 1). This decrease was statistically significant (P = 0.006). Also when airflow cooling was used, ODC activity was decreased statistically significantly (P = 0.041) after 2 h exposure at 6.0 W/kg by 20% in case of the CW exposure. In ECACC cells exposed at 6.0 W/kg for 2 h without airflow, ODC activity was also decreased (Table 2), and the decrease was of similar magnitude (30%) as that observed in the corresponding experiment with ATCC cells. However, the difference was not statistically significant. For the 8 and 24 h exposure durations, no effects on ODC activity were observed in either of the cell lines (Tables 1 and 2).

The results from the experiments with the waveguide exposure chamber (Table 3) differ from those obtained with the Crawford chamber. After 8 h of exposure at 6.0 W/kg, ODC activity of ATCC cells was increased by 45% for the CW signal and by 43% for the DAMPS signal. This difference was statistically significant for the DAMPS signal (P = 0.014).

Comparison of Tables 1 and 2 does not show any obvious differences in responses to RF radiation between L929 cells purchased from different cell banks (ATCC and ECACC). The only clear effect was reduction of ODC activity after 2 h of exposure at high SAR (6 W/kg) without air flow cooling. This effect, which is probably related to heating, was of similar magnitude in ECACC (30%) and ATCC (33%) cells but statistically significant only in the latter. The data is not strong enough to conclude that the effect is stronger in ATCC cells. However, appearance of the cultured cells was different: at equal densities ATCC cells were more rounded than ECACC cells. Also ODC activities of control ATCC cells were slightly lower than those of control ECACC cells.

5 Discussion

Increase in cellular ODC activity has been reported to be involved in carcinogenesis [O'Brien et al., 1982; Auvinen et al., 1992] and several tumor promoters induce cellular ODC activity [O'Brien and Diamond, 1977]. Reports of increased ODC activity after RF radiation exposure [Byus et al., 1988; Litovitz et al., 1993, 1997; Penafiel et al., 1997] have raised concern about the possible carcinogenicity of RF radiation. Enhancement of ODC activity per se is not sufficient to induce tumors but increased ODC activity may serve as a mechanism for tumor promotion [Clifford et al., 1995]. However, the increase of ODC activity in cell cultures induced by known tumor promoters is up to 20–30 times [O'Brien and Diamond, 1977; Azadniv et al., 1995], which is much higher than the 1.4–1.9-fold changes reported after RF radiation [Byus et al., 1988; Litovitz et al., 1993, 1997; Penafiel et al., 1997]. In any case, increased ODC activity after RF radiation exposure may serve as an indicator of biological

Table 3. ODC activities of L929 cells of ATCC origin after 8 h sham-sham experiments or various exposures at SAR level of 6 W/kg in waveguide exposure chamber. Column headings and abbreviations are as indicated in Table 1.

Modulation	n (C/E)	Mean C	Mean E	F	(p) p	ODC activity ratio
sham-sham	6/6	113.3 ± 8.7 (12.3)	113.6 ± 15.1 (12.3)	0.00	(0.988) 0.999	1.01 ± 0.14
CW	10/10	73.2 ± 12.5 (16.8)	105.4 ± 13.6 (16.8)	6.37	(**0.024**) 0.070	1.45 ± 0.16
DAMPS	10/10	82.5 ± 12.4 (18.6)	128.5 ± 16.1 (18.6)	11.24	(**0.005**) **0.015**	1.43 ± 0.23

interaction between RF energy and living cells.

The present study was carried out to replicate the results of Penafiel et al. [1997], who reported increased ODC activity in L929 cells after exposure to DAMPS modulated RF radiation. Furthermore, we compared L929 cells purchased from two cell banks (ATCC and ECACC) and two types of exposure setups. We were unable to confirm the induction of cellular ODC activity in L929 cells after exposure to DAMPS modulated radiofrequency radiation carried out in a Crawford chamber. L929 cells from either ATCC or ECACC did not show increased ODC activity in response to DAMPS modulated or unmodulated RF fields at SAR levels of 2.5 or 6 W/kg. In contrast, some evidence of decreased ODC activity was seen at the higher exposure level.

In their attempt to replicate results of Penafiel et al. [1997], Desta et al. [2003] reported results that were quite consistent with the present study. They exposed L929 cells in a Crawford chamber for 8 h to DAMPS modulated RF radiation at SAR levels from < 1 to 15 W/kg and no enhancement of ODC activity was observed. On the contrary, Desta et al. reported a decrease in cellular ODC activity at SAR levels over 6 W/kg producing over 1 °C temperature rise in the cell culture medium. ODC activity was decreased by 25% after exposures at 6–10 W/kg. In the present study, a decrease in ODC activity of the same magnitude was detected after 2 h of exposure to DAMPS modulated RF radiation at 6 W/kg in a Crawford chamber without airflow. ODC activity was decreased by 33% in L929 cells of ATCC origin and by 30% in cells of ECACC origin. In these exposures, the expected temperature rise was 1.14 °C. The expected temperature rise of 0.28 °C may also explain the small (11–20%) decreases of ODC activity seen in ATCC cells after 2 h exposures at 6 W/kg with airflow. In our previous study [Höytö et al., 2006], we have shown that 2 h exposure to a 1.1 °C increase in temperature by conventional heating (no RF radiation involved) in an incubator decreased cellular ODC activity in L929 cells by 43%. These findings confirm that precise temperature control of the exposed cultures is essential in ODC activity studies.

There were some discrepancies between the protocols we and Penafiel et al. [1997] used, that may have caused the differences in results. Our Crawford chamber exposure system was slightly modified to enhance the dosimetry and to enable us to control the temperature of the cell cultures. Furthermore, the exposure system allowed us to compare between exposures with and without air cooling. Apart from these modifications, the exposure chamber was essentially similar to that used by Penafiel et al. [1997] and it is not likely that the differences in the results are due to these modifications. The ODC activity assays were also somewhat different. Our method was apparently more sensitive, since we

were able to make the assay with cells from a single flask. However, differences in the ODC assay methods should not cause disparity in the outcome. If anything, with the more sensitive method we should have been able to observe smaller differences in ODC activity between the samples, if they were present. We used antibiotics in the cell culture medium, which was not reported by Penafiel et al. [1997]. Antibiotics are generally used in cell culture to avoid uncontrolled microbial growth and the related adverse effects, including reduced reproducibility of experimental results. In principle, presence of antibiotics might alter cellular responses to other agents, but there are no data about antibiotics-related changes in sensitivity to RF radiation.

It is of interest that, in contrast to the experiments with the Crawford chamber, ODC activity was increased in the cells exposed at 6 W/kg in the waveguide chamber. There is no obvious reason why RF fields would have different effects in different exposure chambers. The most important difference between these two exposure chambers is the method of temperature control. The waveguide chamber utilizes more efficient water cooling compared to air cooling in the Crawford chamber. Thus, the temperature rise that occurs in the Crawford chamber during exposure is the most likely explanation for the decrease in ODC activity seen after 2 h exposures, as discussed above. The increased ODC activity seen in the waveguide chamber is more difficult to explain. We offer two alternative hypotheses. The first hypothesis is that RF field exposure for 8 h at 6 W/kg increases ODC activity if temperature is kept constant. This effect is observable in the waveguide experiments because of the efficient temperature control, but is not seen in the 8 h experiments with the Crawford chamber, because the effect is cancelled by the suppressing effect of increased temperature on ODC activity. The second hypothesis is related to the adjustment of temperature control in the waveguide chamber. The temperature of the circulating water is adjusted for each experiment according to the SAR level used, so that temperature of the cell culture medium is kept constant independently of SAR level. If the cells (which are attached to the bottom of the cell culture dish and therefore closest to the cooling water) experience a lower temperature than the average temperature of the medium, and if we assume that lower temperature results in higher ODC activity, this might explain the increased ODC activities observed. The results of the present study do not allow for concluding which of the two hypotheses is correct. Additional studies are warranted to investigate possible increase of ODC activity in non-thermal exposure conditions. It is noteworthy that the ODC increases observed in the waveguide chamber were of similar magnitude in response to DAMPS and CW signals. Thus, if the findings of the present study reflect a true non-thermal effect, the findings are not consistent with the studies reporting that only modulated signals affect ODC activity [Litovitz et al., 1993; Penafiel et al., 1997].

In the present study, we compared L929 cells purchased from two cell banks, ATCC and ECACC, and there were no differences between them. Hence, possible subtle dissimilarity between cells from different cell banks did not yield two distinct responses to RF radiation exposure.

In summary, we investigated effects of continuous wave and DAMPS modulated RF electromagnetic fields on cellular ODC activity in L929 cells. The present study did not confirm previous results reporting increased ODC activity in cells exposed to modulated RF fields. When an exposure system similar to that used in the previous studies (Crawford cell) was used, ODC activity was either not affected (in case of

8 or 24 h exposures) or decreased after a 2 h exposure at the highest SAR level (6 W/kg). The decrease was statistically significant only for L929 cells purchased from ATCC and most pronounced when cooling with air flow was not used, and is most likely related to increased temperature. However, different results were obtained in experiments with another exposure system with more efficient temperature control. In this exposure system, ODC activity was increased after 8 h of exposure at 6 W/kg when the temperatures of the exposed and control cultures were kept identical. Further studies are warranted to explore whether this finding reflects a true non-thermal effect. No differences were seen between the CW and DAMPS signals in any of the experiments, so the present study did not provide evidence for modulation-specific effects reported in some earlier studies.

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Chapter 4.

Ornithine decarboxylase activity is affected in primary astrocytes but not in secondary cell lines after RF radiation exposure at 872 MHz

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1 Abstract

Purpose: The effects of radiofrequency (RF) radiation on cellular ornithine decarboxylase (ODC) activity were studied in fibroblasts, two neural cell lines and primary astrocytes. Several exposure times and exposure levels were used, and the fields were either unmodulated or modulated according to the characteristics of the Global System for Mobile (GSM) communications.

Materials and methods: Murine L929 fibroblasts, rat C6 glioblastoma cells, human SH-SY5Y neuroblastoma cells, and rat primary astrocytes were exposed to RF radiation at 872 MHz in a waveguide exposure chamber equipped with water cooling. Cells were exposed for 2, 8, or 24 hours to continuous wave (CW) RF radiation or to a GSM type signal pulse modulated at 217 Hz, at specific absorption rates of 1.5, 2.5, or 6.0 W/kg. Cellular ODC activities of cell samples were assayed.

Results: ODC activity in rat primary astrocytes was decreased statistically significantly (p values from 0.003 to <0.001) and consistently in all experiments performed at two exposure levels (1.5 and 6.0 W/kg) and using GSM modulated or CW radiation. In the secondary cell lines, ODC activity was generally not affected.

Conclusions: ODC activity was affected by RF radiation in rat primary neural cells, but the secondary cells used in this study showed essentially no response to similar RF radiation. In contrast to some previous studies, no differences between the modulated and continuous wave signals were detected. Further studies with primary astrocytes are warranted to confirm the present findings and to explore the mechanisms of the effects.

2 Introduction

Alterations in cellular ornithine decarboxylase (ODC) activity are related to cellular growth and differentiation. Increase in ODC activity has been reported to occur for example in cancerous cells. Overexpression of ODC by itself is not sufficient to induce tumours in normal cells, but increase in cellular ODC activity enhances tumour development in initiated premalignant cells (Clifford et al. 1995). Reports of increased ODC activity in cell cultures exposed to radiofrequency (RF) radiation (Byus et al. 1988, Litovitz et al. 1993, 1997, Penafiel et al. 1997) have raised concern about possible health effects, as use of mobile telephones has rapidly expanded. However, compared to the strong ODC activation caused by known chemical tumor promoters, enhancement in ODC activity in RF radiation exposed cells has generally been quite low, from 20% (Penafiel et al. 1997) to 90% (Litovitz et al. 1993), and not all studies

have been able to show the effect at all (Desta et al. 2003). In several studies, the reported increase in ODC activity was associated with amplitude-modulated RF radiation, and no effects were seen with non-modulated (continuous wave [CW]) radiation at similar exposure levels (Litovitz et al. 1993, 1997, Penafiel et al. 1997). Such modulation-specific effects would be particularly relevant to mobile phone exposure, as amplitude-modulated signals are used in digital mobile phone systems. The World Health Organization (WHO) has recommended further studies to explore effects of RF radiation on cellular ODC activity (Repacholi 1998).

We have previously reported that exposure to low-level (SAR levels of 0.2 or 0.4 W/kg) RF radiation using CW signals or modulation similar to that used in the global system mobile (GSM) mobile phone system (217 Hz) did not affect cellular ODC activity in L929 cells (Höytö et al. 2006). The present study examined effects of CW or GSM modulated RF radiation on cellular ODC activity at higher exposure levels (SAR levels of 1.5, 2.5, and 6.0 W/kg). Exposures were carried out using murine L929 fibroblasts, which have been used in several other studies, as well as rat C6 glioblastoma, human SH-SY5Y neuroblastoma cell lines, and rat primary astrocytes to study effects on neural cells. Neural cells are of special interest, since mobile phones are usually held in the proximity of the brain tissues and the effects of RF radiation on ODC activity in neural cells have not been studied previously. Moreover, primary cells have been used seldom to study effects of RF radiation, although they are functionally closer to intact tissues than transformed cell lines.

3 Materials and methods

3.1 Secondary cell lines

Three secondary cell lines and primary cultured cells were used for RF radiation exposures. L929 murine fibroblasts (obtained from ECACC, European Collection of Cell Cultures, Salisbury, UK) were cultured as described previously (Höytö et al. 2006) and C6 rat glioblastoma cells (obtained from Dr Nikolaus Plesnila, University of Munich, Germany) were cultured in Dulbecco's Modified Eagle Medium (DMEM) and SH-SY5Y human neuroblastoma cells (obtained from Dr Sven Påhlman, University of Uppsala, Sweden) in DMEM with Glutamax I, 4500 mg/l D-glucose, both supplemented with 10% heat-inactivated fetal bovine serum, penicillin (50 U/ml), and streptomycin (50 mg/ml) (all obtained from Gibco, Invitrogen, Carlsbad, CA, USA). Cells were harvested by trypsinisation (trypsin, Gibco, Invitrogen, Carlsbad, CA, USA). Approximately 20 h prior to exposure, cells were seeded into four 55 mm diameter glass Petri dishes (Schott, Mainz, Germany). To provide sub-confluent cultures for exposures, L929 cells were seeded at a density 2 x 10^6 cells/dish for shorter (2 and 8 h) exposures and at a density 1 x 10^6 cells/dish for 24 h exposures in 5 ml of culture medium. Cell densities for C6 cells were for 2 and 8 h exposures 1.5 x 10^6 cells/dish and for 24 h exposures 0.75 x 10^6 cells/dish. For SH-SY5Y cells the density was 4 x 10^6 cells/dish for all of the exposures.

3.2 Primary cells

Primary rat astrocyte cultures were derived from the brains of 2-days-old rat. Briefly, Wistar neonatal rats were sacrificed by decapitation and brains were isolated. The cerebral cortices were excised from the medulla and meninges and blood vessels were removed carefully. The refined cortical tissue was minced into small pieces with a scalpel. Cells were separated by dispase treatment with 150 mg dispase (Gibco, Invitrogen, Carlsbad, CA, USA) in 50 ml phosphate buffered saline (PBS) w/o Ca^{2+}, Mg^{2+} (Oy Reagena Ltd, Kuopio, Finland). During the

dispase treatment, the tissue was stirred (300 rpm, 30 °C) in 5–10 ml of dispase for 5 min and cleared solution was filtered through 100 mm nylon net filter (Millipore, Carrigtwohill, Ireland) and 5 ml of DMEM with 580 mg/l L-glutamine, containing 20% FBS, 50 mg/ml streptomycin, and 50 U/ml penicillin (all obtained from Gibco, Invitrogen, Carlsbad, CA, USA) was added. Dispase treatment was repeated until all of the dispase had been consumed. Then the solution containing dissolved tissues were centrifuged at 160 *g* for 5 min. The tissue sediments were triturated with DMEM. The cells were cultured in 75 cm^2 tissue culture flasks (Nunc A/S, Roskilde, Denmark) in 15 ml of medium. Flasks for pure astrocyte cultures were shaken vigorously before each medium (20 ml of DMEM with 10% FBS) change, which was done first after 2 days and then after every 4 days altogether 2–3 times. The purified astrocytes were detached by trypsinization and used for exposures at passage numbers 5–10. For exposures, 1.25 x 10^6 cells/dish (2 and 8 h) and 1.0 x 10^6 cells/dish (24 h) were plated into glass dishes 20 h prior to the exposure. The study plan for the preparation of rat primary astrocyte cultures was approved by the Research Animal Use Committee of the University of Kuopio.

3.3 Exposure

At the beginning of the exposures, cells were evenly distributed in the dishes and two randomly selected dishes were placed in the exposure chamber and the other two in the sham-exposure chamber. To avoid excess activation of ODC, the medium was never changed prior to the exposure. Exposures were carried out in a waveguide exposure system described earlier together with dosimetric details (Höytö et al. 2006). The exposure system involves a water circulation system for controlling the temperature of the cell culture dishes. In spite of the temperature control, some of the experiments reported in this paper were carried out with a slight temperature difference between the exposure and sham-exposure chambers (maximally 0.8 °C lower in the latter), due to an improperly functioning temperature sensor. This error was detected and corrected after the initial experiments (all experiments with L929 cells and 1.5–2.5 W/kg experiments with C6 and SH-SY5Y cells), and temperatures of the chambers were equal in the rest of the experiments. The effects of the small temperature difference are commented in the results and discussion sections. L929, C6, and SH-SY5Y cells were exposed to 872 MHz RF radiation for 2, 8, and 24 h at SAR levels of 1.5, 2.5, and 6.0 W/kg with or without modulation (pulse frequency 217 Hz; GSM-type modulation). Primary rat astrocytes were exposed for equal times and exposure levels, except for SAR level of 2.5 W/kg, which was not used.

3.4 ODC activity assay

The medium was removed from the dishes after exposure and the cells were washed twice with ice-cold (4 °C) Ca^{2+}- and Mg^{2+}-free PBS. The cells were detached by scraping in 1 ml of ice-cold PBS. The cell suspension was centrifuged (500 *g*, 5 min, 4 °C) and the supernatant was discarded. Dry cell pellets were frozen in –70 °C for ODC activity analysis to be performed later.

ODC activity of coded samples was assayed in a blinded manner according to the method described by Jänne and Williams-Ashman (1971) with some modifications. Briefly, frozen cell samples were lysed in buffer containing 25 mM Tris pH 7.4 (Trizma Base, Sigma-Aldrich ChemieGmbh, Steinheim, Germany), 0.1 mM ethylenedinitrilo-tetraacetic acid, disodium salt dihydrate (EDTA, Titriplex III, Merck KGaA, Darmstadt, Germany), 0.1% Triton X-100 (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany), and 1 mM DL-dithiothreitol (DTT, Sigma-Aldrich Chemie Gmbh, Steinheim,

Germany) and vortexed. Homogenate was centrifuged (16,060 *g*, 20 min, 4 °C) and an aliquot of supernatant was incubated using a shaking water bath (Memmert WB 22 & SV 1422, Schwabach, Germany) for 1 h at 37 °C in reaction mixture containing 100 mM Tris pH 7.4, 4 mM EDTA, 4 mM DTT, 0.2 mM L-ornithinmonohydrochlorid (Merck KGaA, Darmstadt, Germany), 0.2 mCi/assay ^{14}C-L-ornithine (specific activity 52.0 mCi/mmol, Amersham Biosciences, Buckingshamshire, UK), and 0.4 mM pyridoxal-5-phosphate (PLP, Sigma-Aldrich Chemie Gmbh, Steinheim, Germany). $^{12}CO_2$ was liberated by addition of 2 M citric acid (Riedel-de Haën, Sigma-Aldrich, Seelze, Germany), and incubation was continued for 15 min. Liberated $^{14}CO_2$ was trapped in pleated filter paper (Chromatography paper 3MM Chr, Whatman International Ltd, Maidstone, UK) wetted with Solvable (Packard Instruments, Groningen, The Netherlands). Filter papers were transferred to scintillation vials (Perkin Elmer Life Sciences, Wallac Oy, Turku, Finland) containing 3 ml OptiPhase HiSafe 3 scintillant (PerkinElmer Life Sciences, Fisher Chemicals, Loughborough, UK) and the activity was measured using a liquid scintillation counter (Wallac WinSpectral 1414, PerkinElmer Life Sciences, Wallac Oy, Turku, Finland). Protein content of the samples was measured using Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

Units of ODC activity were expressed as pmol $^{14}CO_2$ generated/h/mg protein at 37 °C ± SEM (n = 5–12). Due to day-to-day variation of ODC activity in control cultures results are expressed also as ODC activity ratio ± SEM (n = 3–6), which is calculated by dividing the mean ODC activity of the two simultaneously exposed cultures by the mean of the two non-exposed control cultures.

3.5 Positive controls

Positive control experiments were carried out to prove the responsiveness of the cell lines used in the studies. Cells were plated in two dishes containing medium with serum and in two dishes containing medium without serum and incubated for 20 h. Fresh medium (with serum) was changed to one dish per group and all dishes were further incubated for 4 h. Cellular ODC activity of frozen cell samples was assayed.

3.6 Sham-sham experiments

Sham-sham experiments were carried out to check possible differences between the exposure chambers. In sham-sham experiments no RF radiation was applied and cells were placed in the chambers for 2, 8, and 24 h at equal cell densities as in real exposure experiments. This procedure was repeated with C6 and SH-SY5Y cells after calibration of the temperature sensors of the water circulation system, as some exposures were carried out after the calibration.

3.7 Statistics

Statistical analyses were carried out using SPSS for Windows release 11.5.1 (SPSS Inc., Chicago, Illinois, USA). ODC activity values were used for linear mixed model analysis to obtain an overall p value for all exposure times of a given exposure level. If the overall p value was significant ($p \leq 0.05$), additional analyses were performed as post-tests to identify significant p values at single exposure times. Treatment and exposure time (in the overall analysis) were used as a fixed factors and replicate as a random factor in the analysis. A replicate consisted of two dishes exposed and two dishes sham-exposed simultaneously. Random factor was included in the model since the ODC activities of the control cultures varied from day to day.

Table I. Cellular ODC activity in murine L929 fibroblasts exposed to continuous wave (CW) or GSM-modulated RF radiation at various exposure levels up to 6 W/kg.

Exposure time (h)	n C/E	Control (mean ± SEM)	Exposed (mean ± SEM)	P_1	P_2	ODC activity ratio
Sham-sham						
2	8/8	3686.7 ± 169.0	2685.2 ± 285.5		**<0.001**	0.72 ± 0.06
8	6/6	1983.8 ± 480.0	1996.7 ± 472.5	**0.002**	0.960	1.00 ± 0.05
24	6/6	1480.1 ± 373.9	1224.9 ± 359.5		0.227	0.82 ± 0.10
1.5 W/kg GSM						
2	6/6	5564.2 ± 541.6	4257.8 ± 351.7		0.052	0.78 ± 0.07
8	6/6	2521.6 ± 160.5	2436.8 ± 281.4	**0.003**	0.549	0.95 ± 0.11
24	6/6	2217.4 ± 403.9	1507.6 ± 284.8		**0.020**	0.68 ± 0.05
1.5 W/kg CW						
2	6/6	3710.8 ± 235.1	3510.3 ± 252.5		-	0.95 ± 0.05
8	6/6	2086.3 ± 435.7	2168.1 ± 488.3	0.405	-	1.00 ± 0.15
24	6/6	1417.0 ± 230.8	1268.7 ± 219.3		-	0.89 ± 0.10
2.5 W/kg GSM						
2	5/6	3447.2 ± 130.9	3361.3 ± 425.0		-	0.98 ± 0.13
8	6/6	2772.7 ± 691.8	2840.1 ± 823.6	0.814	-	0.99 ± 0.05
24	6/6	1954.1 ± 156.3	2076.3 ± 349.8		-	1.09 ± 0.33
2.5 W/kg CW						
2	5/6	3514.4 ± 298.7	3227.1 ± 310.3		-	0.90 ± 0.09
8	6/5	2420.9 ± 291.5	2815.3 ± 233.9	0.875	-	1.21 ± 0.10
24	6/6	1193.7 ± 266.3	976.3 ± 105.9		-	0.89 ± 0.19
6.0 W/kg GSM						
2	6/6	4435.4 ± 311.3	3653.8 ± 387.8		-	0.83 ± 0.15
8	6/6	1736.0 ± 106.7	1916.3 ± 157.7	0.934	-	1.10 ± 0.01
24	6/6	795.8 ± 245.1	1344.6 ± 576.6		-	1.46 ± 0.34
6.0 W/kg CW						
2	6/6	5399.5 ± 251.0	4046.9 ± 225.1		**0.004**	0.78 ± 0.02
8	6/6	2202.5 ± 323.1	1924.7 ± 303.1	**0.002**	0.398	0.92 ± 0.13
24	6/6	747.4 ± 137.3	702.6 ± 103.5		0.724	1.01 ± 0.14

n (C/E) = number of control (C) and exposed (E) samples. The ODC activities are expressed as pmol $^{14}CO_2$ generated/60 min/mg protein at 37 °C. P_1 = Overall p value. P_2 = p value for a single exposure time, calculated as a post-test only if the overall p value was significant. Significant p values (≤ 0.05) are indicated with bold font. ODC activity ratio (mean ± SEM) was calculated by dividing the mean ODC activity of the two simultaneously exposed cultures by the mean of the two non-exposed control cultures. Sham-sham = experiments in which both the "exposed" and "control" cultures were sham-exposed, i.e., kept in non-energized exposure chambers.

Table II. Cellular ODC activity in rat C6 glioblastoma cells exposed to continuous wave (CW) or GSM-modulated RF radiation at various exposure levels up to 6 W/kg.

Exposure time (h)	n C/E	Control (mean ± SEM)	Exposed (mean ± SEM)	P_1	ODC activity ratio
Sham-sham					
2	6/6	2467.5 ± 462.1	2413.0 ± 398.8		0.96 ± 0.07
8	6/6	1252.7 ± 101.1	728.3 ± 58.2	0.155	0.59 ± 0.08
24	6/6	1122.5 ± 402.5	1083.2 ± 343.0		0.98 ± 0.19
1.5 W/kg GSM					
2	10/10	5527.4 ± 688.0	4875.4 ± 568.7		0.90 ± 0.08
8	6/6	3239.3 ± 981.6	2863.1 ± 626.5	0.075	1.00 ± 0.17
24	6/6	1565.2 ± 260.8	1467.9 ± 257.6		1.00 ± 0.17
1.5 W/kg CW					
2	6/6	4759.6 ± 964.9	4114.4 ± 993.6		0.85 ± 0.10
8	8/8	4111.9 ± 271.2	4259.7 ± 474.5	0.452	1.02 ± 0.13
24	8/8	1420.4 ± 305.8	1540.8 ± 333.2		1.03 ± 0.06
2.5 W/kg GSM					
2	5/6	3750.0 ± 1244.7	3143.4 ± 1124.5		1.08 ± 0.13
8	6/6	2341.9 ± 495.8	2286.2 ± 481.0	0.591	0.98 ± 0.01
24	6/6	1102.8 ± 517.7	1075.8 ± 427.6		1.36 ± 0.30
2.5 W/kg CW					
2	6/6	6081.1 ± 611.0	5999.7 ± 574.9		0.99 ± 0.06
8	6/6	3811.7 ± 683.4	4180.7 ± 884.6	0.599	1.05 ± 0.20
24	6/6	1872.0 ± 656.7	1973.3 ± 565.8		1.33 ± 0.25
Sham-sham					
2	8/8	2928.8 ± 668.8	3279.1 ± 765.1		1.09 ± 0.08
8	6/6	2105.7 ± 586.3	1855.7 ± 533.5	0.924	0.90 ± 0.07
24	8/10	502.6 ± 194.8	523.2 ± 144.5		1.24 ± 0.29
6.0 W/kg GSM					
2	8/8	3019.6 ± 485.7	2634.4 ± 396.8		0.88 ± 0.06
8	8/8	1430.6 ± 276.8	1348.0 ± 459.1	0.073	0.84 ± 0.17
24	11/12	861.4 ± 230.1	570.4 ± 115.7		0.81 ± 0.25
6.0 W/kg CW					
2	6/6	3307.7 ± 408.8	2952.8 ± 436.6		0.88 ± 0.05
8	6/6	5106.6 ± 488.2	5055.4 ± 657.6	0.096	0.99 ± 0.02
24	10/10	1434.1 ± 450.0	928.6 ± 374.9		0.59 ± 0.10

Explanations as in Table I.

4 Results

4.1 Positive controls

Positive control experiments showed that all cell lines used in the present study were responsive to medium change and serum deprivation treatments. Compared to controls, ODC activity after changing of fresh medium was increased by 2.7% in C6 cells, by 54.2% in SH-SY5Y cells, and by 37.4% in rat primary astrocytes. Serum deprivation decreased ODC activity by 87.0% in C6 cells, by 87.6% in SH-SY5Y cells, and by 97.2% in rat primary astrocytes, when compared to corresponding controls. Changing of fresh medium to serum deprived cultures enhanced the ODC activity very strongly in all cells, by 213.7% in rat C6 glioblastoma cells, by

708.9% in rat primary astrocytes, and by 977.2% in human SH-SY5Y neuroblastoma cells. The results of positive control experiments for L929 cells were published earlier (Höytö et al. 2006).

4.2 Sham-sham experiments

A total of six sham-sham comparisons with no RF radiation applied were performed. In one such experiment with L929 cells, a statistically significant difference between the two exposure chambers was observed after 2 h of exposure (Table I). This difference is most likely related to the small temperature difference between the exposure chambers (see Materials and methods). No statistically significant differences were observed in the other sham-sham experiments (Tables II–IV).

4.3 Secondary cell lines

The results presented in Tables I–III show that cellular ODC activity in secondary cell lines was generally not affected by RF radiation at low SAR levels. The only significant difference below 6 W/kg was observed in murine L929 fibroblasts exposed to the GSM signal at 1.5 W/kg (Table I). This significant overall p value (0.003) resulted mainly from the 32% reduction of ODC activity at the 24 h time point ($p = 0.020$). Like the difference seen in the sham-sham experiment with L929, this difference is most likely related to the small temperature difference between the exposure chambers. Also exposure to the CW signal at 6.0 W/kg resulted in a significant difference ($p = 0.002$) in L929 cells, mainly related to a 22% decrease of ODC activity after 2 h of exposure. In rat C6 glioblastoma cells, ODC activity was not altered statistically significantly at any time point after any kind of exposure, although there was a tendency towards decreased ODC activities at 6 W/kg (Table II). In the human SH-SY5Y neuroblastoma cell line, exposure to CW RF radiation at 6.0 W/kg resulted in a statistically significant p value of 0.002 (Table III), associated with a 15% decrease of ODC activity after 2 h of exposure.

4.4 Primary cells

In experiments with rat primary astrocytes, statistically significant differences were found at all exposure levels and signals, with overall p values from < 0.001–0.003 (Table IV). ODC activities were generally decreased (16 to 59%, statistically significant in 5 out of 8 cases) after 2 or 8 h of exposure, but the 24 h ODC activity was decreased (41%, $p = 0.042$) only in cells exposed to the GSM signal at 6 W/kg.

5 Discussion

Exposure to radiofrequency radiation similar to that emitted by mobile phones has been reported to enhance cellular ODC activity in cell cultures. In murine L929 fibroblasts, ODC activity increased by 40% after exposure to 840 MHz TDMA modulated RF radiation at 2.5 W/kg for 8 h (Penafiel et al. 1997). Byus and his co-workers (1988) exposed Reuber H35 hepatoma, Chinese hamster ovary, and human 294 T melanoma cells to 450 MHz RF fields sinusoidally amplitude modulated at 16 Hz, at a power density of 1.0 mW/cm^2 (peak envelope power) for 1 h and observed a 50% increase in ODC activity. However, in an attempt to replicate the study of Penafiel et al. (1997), Desta et al. (2003) failed to show any increase in ODC activity in L929 cells after 8 h of exposure to 835 MHz RF radiation with modulation typical to TDMA mobile phones at SAR levels from < 1 W/kg to 15 W/kg. In our previous study, we also were unable to show enhancement of ODC activity in L929 cells exposed for 2–24 h to 872 MHz continuous or GSM modulated RF radiation at SAR levels of 0.2–0.4 W/kg (Höytö et al. 2006).

Table III. Cellular ODC activity in human SH-SY5Y neuroblastoma cells exposed to continuous wave (CW) or GSM-modulated RF radiation at various exposure levels up to 6 W/kg.

Exposure time (h)	n C/E	Control (mean ± SEM)	Exposed (mean ± SEM)	P_1	P_2	ODC activity ratio
Sham-sham						
2	5/6	1648.9 ± 255.1	1917.0 ± 247.8		-	1.07 ± 0.04
8	6/6	311.8 ± 44.8	206.7 ± 38.2	0.951	-	0.64 ± 0.07
24	6/6	204.0 ± 29.1	189.3 ± 56.4		-	0.86 ± 0.19
1.5 W/kg GSM						
2	6/6	1519.6 ± 237.4	1740.4 ± 327.4		-	1.11 ± 0.08
8	6/6	994.7 ± 180.0	1063.8 ± 132.0	0.165	-	1.14 ± 0.12
24	5/6	109.7 ± 35.6	126.5 ± 30.1		-	1.09 ± 0.15
1.5 W/kg CW						
2	5/6	1953.5 ± 275.5	2259.5 ± 288.4		-	1.22 ± 0.23
8	6/6	534.6 ± 94.5	556.5 ± 79.0	0.137	-	1.08 ± 0.09
24	6/6	328.3 ± 25.9	344.1 ± 25.8		-	1.06 ± 0.09
2.5 W/kg GSM						
2	6/6	1669.9 ± 184.2	1850.2 ± 111.2		-	1.12 ± 0.03
8	6/6	1173.0 ± 303.1	1134.8 ± 309.8	0.410	-	0.96 ± 0.08
24	7/7	147.2 ± 11.1	177.6 ± 36.1		-	1.40 ± 0.23
2.5 W/kg CW						
2	6/6	3989.0 ± 1141.5	3449.5 ± 1101.6		-	0.82 ± 0.06
8	5/6	688.8 ± 138.3	695.8 ± 191.7	0.055	-	1.06 ± 0.13
24	5/6	148.2 ± 10.1	109.9 ± 4.7		-	0.65 ± 0.14
Sham-sham						
2	6/6	2021.4 ± 372.6	1959.4 ± 436.3		-	0.93 ± 0.07
8	6/6	521.9 ± 75.2	543.5 ± 56.0	0.797	-	1.07 ± 0.07
24	6/6	113.8 ± 6.2	106.0 ± 17.4		-	0.95 ± 0.22
6.0 W/kg GSM						
2	5/6	1074.2 ± 87.5	1171.6 ± 75.7		-	1.06 ± 0.09
8	6/6	777.9 ± 75.0	828.0 ± 71.2	0.210	-	1.07 ± 0.03
24	8/7	97.8 ± 14.6	81.0 ± 15.8		-	1.18 ± 0.32
6.0 W/kg CW						
2	6/6	1746.2 ± 164.2	1494.3 ± 173.4		**0.012**	0.85 ± 0.04
8	6/6	325.6 ± 67.0	262.4 ± 58.5	**0.002**	0.264	0.82 ± 0.03
24	8/6	107.1 ± 16.6	96.3 ± 24.7		0.815	1.45 ± 0.42

Explanations as in Table I.

Table IV. Cellular ODC activity in rat primary astrocytes exposed to continuous wave (CW) or GSM-modulated RF radiation at various exposure levels up to 6 W/kg.

Exposure time (h)	n C/E	Control (mean ± SEM)	Exposed (mean ± SEM)	P_1	P_2	ODC activity ratio
Sham-sham						
2	6/6	1604.2 ± 441.3	1247.6 ± 370.3		-	0.84 ± 0.07
8	6/6	1704.0 ± 410.2	1746.1 ± 809.7	0.787	-	1.07 ± 0.44
24	6/6	132.2 ± 28.7	172.3 ± 59.6		-	1.15 ± 0.28
1.5 W/kg GSM						
2	6/6	2764.3 ± 453.3	2088.3 ± 482.9		**0.008**	0.72 ± 0.07
8	6/6	1486.8 ± 202.8	685.1 ± 109.4	**<0.001**	**0.005**	0.47 ± 0.06
24	6/6	49.0 ± 11.4	72.5 ± 35.2		0.440	1.33 ± 0.41
1.5 W/kg CW						
2	6/6	2253.3 ± 274.8	1815.9 ± 189.1		0.219	0.84 ± 0.14
8	6/6	1459.1 ± 163.3	599.9 ± 88.1	**0.003**	**0.001**	0.41 ± 0.00
24	6/6	155.0 ± 50.5	172.6 ± 105.3		0.849	0.96 ± 0.24
6.0 W/kg GSM						
2	6/6	1519.3 ± 371.2	1154.3 ± 401.3		0.158	0.65 ± 0.19
8	6/6	1175.1 ± 204.1	510.6 ± 126.5	**0.002**	**0.017**	0.50 ± 0.14
24	6/6	193.0 ± 79.2	70.5 ± 22.8		**0.042**	0.59 ± 0.24
6.0 W/kg CW						
2	6/6	1447.8 ± 150.4	766.2 ± 138.8		**0.007**	0.56 ± 0.18
8	6/6	174.4 ± 40.6	123.2 ± 33.5	**0.001**	0.170	0.74 ± 0.16
24	6/6	44.6 ± 16.4	26.4 ± 4.4		0.230	0.91 ± 0.32

Explanations as in Table I

The results of the present study, consistently with our previous findings and those of Desta et al. (2003), did not show enhancement of ODC activity. Instead, a decrease in ODC activity was observed in some cases. Especially primary astrocytes were sensitive to RF radiation and ODC activity was suppressed as much as 59% in those cells. The results from experiments with primary astrocytes are clearly different from those with secondary cells, suggesting a cell type-dependent difference in sensitivity. Sensitivity of primary astrocytes to RF radiation is an interesting finding, as primary cells are not transformed and their function is closer to intact animal tissues than that of secondary cell lines. ODC activity of primary cells after RF radiation exposure has not been previously studied. Studies with whole animals have reported increased ODC activity in brain tissue after long-term (35 d) exposure to 16-Hz modulated 112 MHz fields at 0.75 W/kg (Paulraj et al. 1999) and to 2.45 GHz continuous-wave fields at 0.1 W/kg (Paulraj & Behari 2002), but no effects after short (2 h) exposure to 1.6 GHz pulse modulated RF field (Stagg et al. 2001).

Secondary cell lines were in general not affected by the RF exposure used in the present study. The results from the experiments with L929 cells were probably affected by the small initial temperature difference between the chambers (see Materials and methods), which is likely to explain the reduced ODC activity seen in the sham-sham experiment with this cell line. Our previous results have clearly shown that even small increase in temperature can result in decreased ODC activity (Höytö et al. 2006). Results from the other experiments with L929

cells do not show any differences clearly larger than those seen in the sham-sham experiment, so the two statistically significant *p*-values are also most likely explained by the small temperature difference. Apart from the differences seen in L929 cells, the only statistically significant difference in secondary cell lines was observed in SH-SY5Y cells exposed to the CW signal at 6 W/kg. This might be interpreted as an indication of an effect at high exposure level, but it might also be a chance finding, as many statistical comparisons were performed. The latter alternative is supported by the fact that no effect was observed at the same exposure level with the GSM signal.

All results from this study (reduced ODC activity in primary astrocytes and essentially no effects in secondary cell lines) were similar in experiments with the GSM and CW signals. Hence, the present study did not support the findings of the previous studies in which only modulated signal affected ODC activity.

The increased ODC activity reported in some earlier studies has been considered as an indication of potential harmful health effects. However, also decreased ODC activity (such as that seen in primary astrocytes in the present study) could lead to adverse effects on cells. Decrease in cellular ODC activity leads to impairment in polyamine synthesis and results in decreased proliferation. Inhibition of ODC may activate or prevent apoptosis, programmed cell death, depending on the cell type (Seiler & Raul 2005). It has also been suggested that polyamines act as free radical scavengers and therefore play a role in protecting cells from oxidative stress (Ha et al. 1998). Therefore, a decrease in ODC activity could impair cellular capability to protect DNA from free radical attack.

6 Conclusions

Decrease in cellular ODC activity was detected in rat primary astrocytes after exposure to 872 MHz RF radiation at SAR levels of 1.5 and 6 W/kg. Secondary cell lines, in contrast, showed very little evidence of any effects. This is a very interesting finding, since function of the primary cells is closer to normal tissues than that of secondary transformed cell lines. There are no previous studies on ODC activity in primary cells exposed to RF radiation. Further studies with primary astrocytes are warranted to confirm the present findings and to explore the mechanisms of the effects and their possible relevance to human health.

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Chapter 5.

Radiofrequency radiation does not significantly affect ornithine decarboxylase activity, proliferation, or caspase-3 activity of fibroblasts in different physiological conditions

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Submitted.

Abstract

Purpose: The aim of this study was to test the hypothesis that variations in the physiological state of cells explain inconsistent results from *in vitro* studies on biological effects of radiofrequency (RF) radiation.
Materials and methods: Murine L929 fibroblasts stimulated with fresh medium, stressed with serum deprivation or not subjected to stimulation or stress were exposed in a waveguide exposure chamber to 872 MHz continuous wave or pulse modulated (217 pulses per second) RF radiation at specific absorption rate of 5 W/kg. Ornithine decarboxylase (ODC) activity after 1 and 24h exposures, proliferation during 48 hours after 24h exposure, and caspase-3 activity (a measure of apoptosis) after 1h exposure were measured.
Results: The cells responded to fresh medium and serum deprivation, but no consistent effects of RF radiation were found. One statistically significant (p = 0.03) RF radiation-related difference was observed in ODC activity, but this is most likely a chance finding, as many statistical comparisons were performed, and the finding was not supported by any other data.
Conclusions: The results did not support effects on the endpoints studied. Furthermore, stressed and stimulated cells were not more sensitive than normal cells to possible RF radiation induced effects.

Introduction

Wide-spread use of mobile phones has raised public concern about possible health effects of radiofrequency (RF) radiation. To assess the possible biological effects of weak RF radiation, several *in vitro* studies have evaluated effects on basic cellular processes, such as cellular ornithine decarboxylase (ODC) activity, proliferation, and apoptosis (programmed cell death). Changes in these processes are of interest because of their relevance to the development of cancer. Uncontrolled cell proliferation is characteristic to cancer cells, and removal of mutated cells by apoptosis is an important protection mechanism against cancer in multicellular organisms.

ODC, the first and rate limiting enzyme in polyamine biosynthesis, has been recognized as a significant regulator of cell proliferation. Imbalance in ODC activity may result in uncontrolled growth of transformed cells leading to malignancy (Auvinen *et al*. 1992). Altered cellular ODC activity has also been associated with apoptosis (Thomas & Thomas 2001). Certain chemical tumour promoters are able to enhance ODC activity by 200 - 300 % (O'Brien & Diamond 1977, Azadniv *et al*. 1995). Reports of RF radiation-induced alterations in cellular ODC activity were in the range of 40 - 90 % in murine L929

fibroblasts, Reuber H35 hepatoma, Chinese hamster ovary, and 294T melanoma cells (Byus *et al*., 1988, Litovitz *et al*. 1993 & 1997, Penafiel *et al*. 1997). Some recent studies were unable to show any effects of RF radiation on ODC activity *in vitro* (Desta *et al*. 2004, Höytö *et al*. 2006) and also a decrease in activity in rat primary astrocytes has been reported (Höytö *et al*. 2007a).

Results on RF radiation effects on cellular proliferation are also controversial. Three studies have reported an increase in cellular proliferation (Cleary *et al*. 1990a&b, Velizarov *et al*. 1999) and three studies have resulted in decreased proliferation (Cleary *et al*. 1996, Kwee & Raskmark 1998, Capri *et al*. 2004b). Several studies did not show any differences in proliferation between control and RF exposed cultures (Krause *et al*. 1991, Stagg *et al*. 1997, Vijayalaxmi *et al*. 1997, Nikolova *et al*. 2005, Lixia *et al*. 2006, Merola *et al*. 2006, Sanchez *et al*. 2006, Scarfi *et al*. 2006, Takashima *et al*. 2006, Chauhan *et al*. 2007).

Caspases (cysteinyl aspartate-specific proteinases) mediate the highly ordered process of apoptotic cell death and activation of caspase-3 results in apoptotic phenotype of the cells (Nicholson & Thornberry 1997). Increased apoptosis after RF exposure has been reported in some studies (Peinnequin *et al*. 2000, Marinelli *et al*. 2004, Caraglia *et al*. 2005), while no effects were found in other studies (for example Joubert *et al*. 2006, Merola *et al*. 2006, Joubert *et al*. 2007).

A possible explanation for the inconsistent findings is that the physiological state of cells has varied in different studies. Cells might respond to a weak stimulus like RF radiation only if they are stressed or activated by other environmental factors. To test this hypothesis, we measured the ODC activity, proliferation, and caspase-3 activity after RF radiation exposure in normal, activated, and stressed L929 cells. Activation of the cells was produced with fresh cell culture medium added prior to the RF radiation exposure, and cells were stressed with serum deprivation for 20 h before exposure to RF radiation.

Materials and methods

Cell culture

Murine L929 cells were purchased from ECACC (European Collection of Cell Cultures, Salisbury, UK). Cells were grown in Dulbecco's Modified Eagle medium (DMEM) (Gibco, Paisley, U.K.), supplemented with 10 % bovine serum product (Fetalclone II, HyClone, Logan, Utah, USA), penicillin (50 U/ml), and streptomycin (50 µg/ml) (Gibco, Paisley, U.K.) at 37 °C in 5 % CO_2 in plastic 75 cm^2 flasks (Nunc, Roskilde, Denmark). Cells were harvested by trypsinisation (trypsin, Gibco, Invitrogen, Carlsbad, CA, USA). Approximately 20 h before medium change, cells were plated in four 55 mm diameter glass Petri dishes (Schott, Mainz, Germany). Generally, 1 x 10^6 cells were seeded in each dish, except for the experiments including serum deprivation. For those, 0.3 x 10^6 cells were seeded in non-treated dishes and 0.75 x 10^6 cells were seeded in dishes destined for serum deprivation. These cell counts were selected to obtain subconfluent dishes at the end of the exposures. Activation of the cells was produced with a change of fresh medium and stressing with a change of fresh medium without serum. Medium change or serum deprivation was performed on two dishes and one of these and one non-treated dish was RF-exposed and the other two were sham-exposed to obtain one control dish, one RF-exposed dish, one treated (medium change or serum deprivation) dish, and one dish with treatment and RF exposure. Cells were evenly distributed in the dishes at the beginning of the exposure.

RF exposure

Exposures to RF radiation were carried out in a waveguide exposure system described earlier (Höytö *et al*. 2006). Briefly, the waveguide exposure system provided a uniform SAR distribution (± 35 %) in cell cultures and a water cooling system enabled exposure in controlled temperature (37.0 ± 0.3 °C). The cell culture dishes were placed on the glass surface of a heat exchanger, and the temperatures of the incoming and outgoing water of the heat exchanger were continuously measured and adjusted by a control unit (constructed by Radiation and Nuclear Safety Authority STUK, Helsinki, Finland) with LM45 Precision Centigrade temperature sensors (SOT-23 T4C, order number LM45CIM3, National Semiconductor Corp., Santa Clara, CA, USA). Carbon dioxide (5 % inside the chambers) and temperature-controlled air was provided by ventilation from a cell culture incubator. Exposures were carried out with an 872 MHz signal at SAR level of 5.0 W/kg for 1 or 24 h using either a continuous wave (CW) signal or modulation similar to that used in GSM mobile phones (pulse modulation at 217 Hz).

Ornithine decarboxylase activity

For 1 h exposure cultures, medium with or without serum was changed for two dishes out of four and cultures were further incubated for 24 h and exposed or sham-exposed to RF radiation for 1 h. For cultures to be exposed or sham-exposed for 24 h, fresh medium (with serum) was changed to two dishes right before the exposure. After RF radiation exposure, cellular ODC activity was assayed using method described in Höytö *et al*. (2007a). Briefly, the cells were pelleted after exposure and the cell pellets were frozen in -70 °C for later analysis of ODC activity. Samples were coded and ODC activity was assayed in a blinded manner. Cell samples were lysed in a buffer containing 0.1 mM ethylenedinitrilotetraacetic acid, disodium salt dihydrate (EDTA, Titriplex III, Merck KGaA, Darmstadt, Germany), 25 mM Tris pH 7.4 , 0.1 % Triton X-100 , and 1 mM DL-dithiothreitol (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany) and the homogenate was centrifuged (16 060 *g*, 20 min, 4 °C) . An aliquot of supernatant was incubated at 37 °C in a shaking water bath for 1 hour in ^{14}C-labeled reaction mixture containing 100 mM Tris pH 7.4, 4 mM EDTA, 4 mM DTT, 0.2 mM L-ornithinmonohydrochlorid (Merck KGaA, Darmstadt, Germany), 0.2 µCi/assay ^{14}C-L-ornithine (specific activity 52.0 mCi/mmol, Amersham Biosciences, Buckingshamshire, U.K.), and 0.4 mM pyridoxal-5-phosphate (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany). $^{14}CO_2$ was liberated with 2 M citric acid and samples were further incubated for 15 min. Liberated $^{14}CO_2$ was trapped in pleated filter paper wetted with Solvable (Packard Instruments, Groningen, Netherlands). Activity of the filter papers was measured by liquid scintillation counter (Wallac Win Spectral 1414, PerkinElmer Life Sciences, Wallac Oy, Turku, Finland). Protein content of the samples was measured using Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). Units of ODC activity were expressed as pmol $^{14}CO_2$ generated/h/mg protein at 37 °C ± SEM (n = 3 - 6). Due to day-to-day variation of ODC activity in control cultures results are expressed also as mean ODC activity ratios ± SEM. which is the mean of the ODC activity ratios calculated for each replication as ODC activity of the exposed culture divided by ODC activity of the corresponding non-exposed culture.

Proliferation

Serum-containing medium was changed to two dishes and cultures were exposed or sham-exposed to RF radiation for 24 h. Four independent replications were performed. Afterwards, proliferation assay was performed using AlamarBlue reagent (BioSource International, Inc., Camarillo, CA, USA) according to the manufacturer's

protocol. Briefly, cells were washed twice with ice-cold (4 ºC) PBS (phosphate buffered saline without Ca^{2+},Mg^{2+}, Oy Reagena Ltd, Toivala, Finland), removed from dishes by scraping in 1 ml of ice-cold PBS. Cells were counted and 1 x 10^4 cells were plated in 450 µl of cell culture medium in 96 microtiter well plates (Nunc, Roskilde, Denmark). Then 50 µl of AlamarBlue was added to each well. Culture medium was used as blank samples. The plates were exposed to an excitation wavelength of 540 nm and the emission at 595 nm was recorded on a fluorometer (Perkin Elmer HTS 7000 Plus Bio Assay Reader, Turku, Finland) at time points 0, 2, 4, 6, 24, and 48 h. In the meantime, the plates were kept in a cell culture incubator. The viability was expressed as a ratio of fluorescence emitted by treated cells compared to control cells. It is noteworthy that measured values at time points under 6 h reflect metabolic activity of the cells, not actual proliferation.

Caspase-3 -like protease activity

Medium without serum was changed to two dishes and cultures were incubated for 24 h before the 1 h exposure or sham-exposure to RF radiation. Three independent replications were performed. After exposure, cells were removed from dishes by scraping and suspended. Cells were centrifuged (380 x *g*, 5 min, 4 °C) and the supernatant was discarded. The cells were washed with 1 ml of PBS and centrifuged (16 000 x *g*, 1 min, 4 °C). The cell pellet was suspended in 100 µl of lysis buffer (pH 7.5) containing 10 mM Trizma Base and 1 % Triton X-100 (both from Sigma-Aldrich Chemie Gmbh, Steinheim, Germany) in PBS. Cell suspension was incubated on ice for 30 minutes and then centrifuged (13 000 x *g*, 45 min, 4 °C). Supernatant was transferred to new vials and kept on ice during the assay. Protein content of the samples was measured using Bio-Rad protein assay kit and the samples were diluted to contain 1 mg protein/1 ml assay buffer. Assay buffer (pH 7.5) contained 10 % glycerol and 2 mM DL-dithiotreitol (both from Sigma-Aldrich Chemie Gmbh, Steinheim, Germany) in 20 mM Hepes buffer (Gibco BRL, Life Technologies Ltd, Paisley, UK). Assay was carried out in 96 well microtiter plates. Each well contained 200 µl of assay buffer, 50 µl of cell suspension, and 50 µl of 120 µM fluorogenic caspase-3 substrate II (DEVD-AMC, Calbiochem, CN biosciences inc., La Jolla, CA, USA). The plates were incubated in dark at 37 °C for 1 h. The plates were exposed to an excitation wavelength of 360 nm and the emission at 460 nm was recorded on the fluorometer.

Sham-sham experiments

Sham-sham experiments were carried out to exclude the possibility of any unknown differences in cell cultivation conditions between the two waveguide exposure chambers. These experiments were otherwise identical with the RF exposure experiments, but both the "RF exposure" chamber and the sham exposure chamber were non-energized.

Statistical analysis

Statistical analyses were carried out using SPSS for Windows release 14.0.1 (SPSS Inc., Chicago, Illinois, USA). Raw values of ODC activity, proliferation, and caspase-3 activity were used for linear mixed model analysis. In the analysis of ODC activity values, two exposure durations were tested separately. Replicate was included as random effect and modulation, RF radiation exposure, treatment and their interactions were investigated as fixed effects. In analysis of proliferation values, an additional random factor "dish" was included to take into account multiple measurements made from same dish and measurement time was included as a fixed factor. A $p < 0.05$ was used as the limit for statistical significance.

Table I. Cellular ODC activity in murine L929 fibroblasts exposed to continuous wave (CW) or GSM-modulated (217 Hz) RF radiation at SAR level of 5 W/kg.

Treatment	n C/E	Non-RF-Exposed (mean ± SEM)	RF-Exposed (mean ± SEM)	*P*	Mean ODC activity ratio
1 h Sham-sham					
none	6/6	4138.6 ± 782.2	3201.7 ± 629.2	0.369	0.77 ± 0.06
fresh medium	3/3	9295.0 ± 1872.7	9821.1 ± 2007.2	0.718	1.08 ± 0.13
serum deprivation	3/3	196.2 ± 83.5	141.7 ± 29.5	0.970	0.86 ± 0.18
1 h CW					
none	6/6	3582.0 ± 526.3	3777.8 ± 416.2	0.718	1.08 ± 0.06
fresh medium	3/3	7310.0 ± 631.2	8581.6 ± 721.5	0.106	1.18 ± 0.05
serum deprivation	3/3	633.0 ± 177.8	639.0 ± 166.7	0.994	1.02 ± 0.10
1 h GSM					
none	6/6	5164.8 ± 659.4	6396.2 ± 849.6	**0.030**	1.24 ± 0.03
fresh medium	3/3	9449.9 ± 1517.2	8847.2 ± 1546.8	0.434	0.94 ± 0.11
serum deprivation	3/3	652.6 ± 242.8	763.8 ± 311.7	0.885	1.12 ± 0.06
24 h Sham-sham					
none	3/3	598.2 ± 179.2	548.1 ± 95.5	0.930	1.14 ± 0.38
fresh medium	3/3	1720.5 ± 690.7	1548.4 ± 329.0	0.765	2.05 ± 1.48
24 h CW					
none	3/3	2016.7 ± 434.0	2814.7 ± 1076.2	0.198	1.30 ± 0.44
fresh medium	3/3	4295.7 ± 1307.6	5121.6 ± 1304.7	0.184	1.24 ± 0.08
24 h GSM					
none	4/4	2611.1 ± 728.4	2679.5 ± 702.7	0.896	1.06 ± 0.10
fresh medium	4/4	3798.7 ± 609.6	4053.6 ± 397.9	0.627	1.11 ± 0.12

n (C/E) = number of control (C) and exposed (E) samples. The ODC activities are expressed as pmol $^{14}CO_2$ generated/60 min/mg protein at 37 °C. Mean ODC activity ratio was calculated as mean (±SEM) of the ratios (exposed/non-exposed) of the original observations, and it is therefore not equal to the ratio of the mean activities shown in the table. Sham-sham = experiments in which both the "exposed" and "control" cultures were sham-exposed, i.e., kept in non-energized exposure chambers. None = No treatments (medium change or serum deprivation) were performed.

Results

Ornithine decarboxylase activity

Sham-sham experiments (no RF radiation applied) showed that there were no differences between the exposure and sham-exposure chambers (Table I). Activation and inactivation treatments were successful in all experiments as addition of fresh medium and serum deprivation produced statistically significantly ($p < 0.05$) different ODC activities in treated L929 cells compared to non-treated cultures. In the experiments with true RF radiation exposure, all but one experiments resulted in slightly higher ODC activity (by 2 – 30 %) in cultures exposed to RF radiation compared to corresponding controls. However, the difference was statistically significant ($p = 0.03$) in only one case out of 15, in cells that were not stimulated or stressed and were exposed to the GSM modulated RF signal for

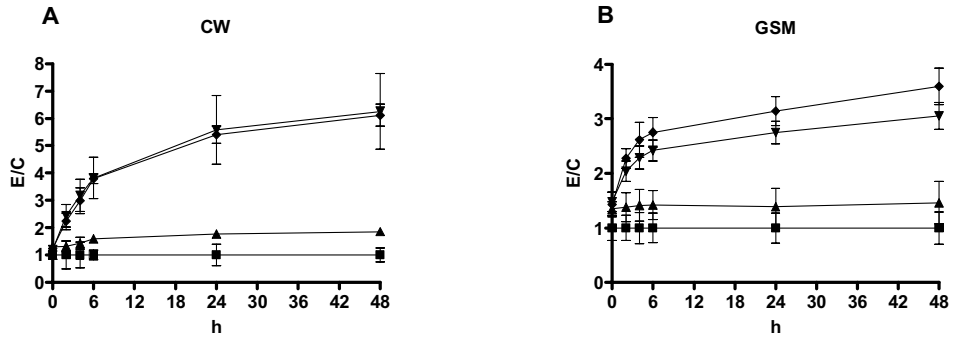

Figure 1. Proliferation in murine L929 fibroblasts exposed to continuous wave (CW) or GSM-modulated (217 Hz) RF radiation at SAR level of 5 W/kg for 24 h. Symbols: ■ control, ▲ exposed to RF radiation, ▼ fresh medium stimulated culture, and ♦ fresh medium stimulated culture exposed to RF radiation. E/C = proliferation of the exposed culture divided by the proliferation of corresponding non-exposed culture (mean ± SEM).

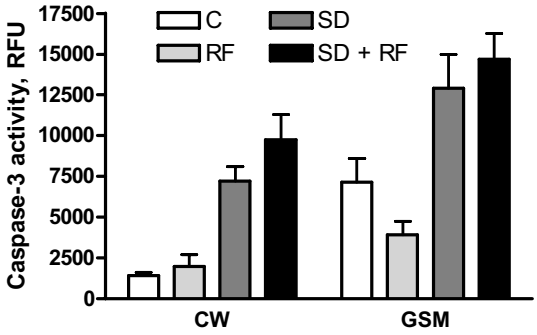

Figure 2. Caspase-3 activity (mean ± SEM) in murine L929 fibroblasts exposed to continuous wave (CW) or GSM-modulated (217 Hz) RF radiation at SAR level of 5 W/kg for 1 h. C = control, RF = exposed to RF radiation, SD = serum deprived culture, SD + RF = serum deprived culture exposed to RF radiation. RFU = relative fluorescence units.

1 h. It is noteworthy that the difference between the two chambers was of the same magnitude in the sham-sham experiments and in the RF experiments.

Proliferation

In sham-sham experiments no statistically significant differences between the exposure and sham-exposure chambers were detected. In all experiments (Figure 1), addition of fresh medium produced significantly ($p < 0.05$) increased proliferation rate during the 48 h follow-up period. In experiments with true RF radiation exposure, proliferation was higher than in the sham-exposed cells in non-stimulated cells exposed to the CW signal, and both in stimulated and non-stimulated cells exposed to GSM signal. However, these differences in proliferation were not statistically significant.

Caspase-3 activity

No statistically significant difference in cellular caspase-3 activity between the exposure and sham-exposure chambers was detected when sham-sham experiments were carried out. Serum deprivation produced

significantly ($p < 0.05$) increased caspase-3 activities in all experiments, as expected (p values not shown). Caspase-3 activity was 35 and 14 % higher after exposures to CW and GSM signals, respectively, than in serum deprived cultures (Figure 2). In cultures without serum deprivation, the activity was 45 % lower after exposure to the GSM signal than in the control culture. However, none of these differences were statistically significant.

Discussion

In this study, exposure to 872 MHz radiofrequency radiation at a SAR of 5.0 W/kg did not induce statistically significant alterations in proliferation after 24 h exposure or caspase-3 activity after 1 h exposure in murine L929 fibroblasts. In the results of the ODC measurements, one significant ($p = 0.03$) difference was found. As this was only one out of 15 statistical comparisons and was not supported by similar differences in stimulated or stressed cells or at another time point, it is most likely a chance finding. Importantly, the results do not support the hypothesis that altered physiological status (stimulation or stress) would alter cellular responses to RF radiation.Controversial results have been obtained from of studies on RF radiation effects on ODC activity in L929 cells. Some reports have indicated increased ODC activity in cells exposed to a 835 MHz Digital advanced mobile phone system (DAMPS, 50 Hz modulated) signal (Litovitz *et al*. 1993 & 1997, Penafiel *et al*. 1997), whereas some studies were not able to confirm these results with or without DAMPS modulation (Desta *et al*. 2003, Höytö *et al*. 2007b), or with CW or GSM signal at 900 MHz (Höytö *et al* 2006) or at 872 MHz (Höytö *et al*. 2007a). Desta and coworkers (2003) reported decreased ODC activity after RF radiation exposure at SAR levels over 6 W/kg, but the temperature increase in cell cultures was shown to cause this effect, and this was further confirmed by our previous work (Höytö *et al*. 2006). The results of the present study support the conclusion that ODC activity in L929 cells is not affected by RF radiation if temperature is kept constant.

In the present study, proliferation was not significantly affected by exposure to RF radiation, although proliferation was slightly higher in the exposed than in the non-exposed samples in three out of four exposure conditions. The result is consistent with previous evidence, as the majority of studies have shown no effects on proliferation (Krause *et al*. 1991, Stagg *et al*. 1997, Vijayalaxmi *et al*. 1997, Nikolova *et al*. 2005, Lixia *et al*. 2006, Merola *et al*. 2006, Sanchez *et al*. 2006, Scarfi *et al*. 2006, Takashima *et al*. 2006, Chauhan *et al*. 2007)

Our results on cellular caspase-3 activity are in agreement with previous studies, as no effects on caspase-3 activity have been reported in human SH-SY5Y neuroblastoma (Joubert *et al*. 2006), human LAN-5 neuroblastoma (Merola *et al*. 2006), or rat primary cortical neuron (Joubert *et al*. 2007) cells exposed to RF radiation. A number of studies have not shown any effects on apoptosis measured by DAPI staining, annexin V-FITC staining, mitochondrial membrane potential, FACS analysis, propidium iodide/YO-PRO-1 staining, or neutral comet assay in RF radiation exposed cells (Port *et al*. 2003, Capri *et al*. 2004a, Hook *et al*. 2004a&b, Nikolova *et al*. 2005, Gurisik *et al*. 2006, Hirose *et al*. 2006, Lantow *et al*. 2006, Sanchez *et al*. 2006, Chauhan *et al*. 2007). However, RF radiation induced increased apoptosis has been reported in a few studies. Survival after agonistic anti-Fas receptor antibody induced apoptosis was decreased in human Jurkat T-cells exposed to a 2.45 GHz CW field at 4 W/kg for 48 h (Peinnequin *et al*. 2000). Marinelli *et al*. (2004) exposed human T-lymphoblastoid leukaemia cells to a 900 MHz CW field at 3.5

mW/kg for 2 - 48 h and activation of both p53-dependent and -independent apoptosis pathways was reported. Annexin V-FITC staining was used to demonstrate increased apoptosis rate in human epidermoid KB cancer cells after exposure to 1.95 GHz CW signal at 3.6 W/kg for 1 - 3 h (Caraglia *et al*. 2005). A recent study reported increased caspase-3 independent apoptosis in rat primary neuronal cultures after exposure to CW 900 MHz RF radiation at SAR level of 2 W/kg for 24 h (Joubert et al. 2008). Markkanen *et al*. (2004) reported increased apoptosis measured by the annexin V–FITC method in mutant yeast cells after coexposure for 1 h to ultraviolet radiation and 872 or 900 MHz GSM modulated (217 Hz) RF radiation at SAR levels of 0.4 and 3.0 W/kg. RF radiation alone (without ultraviolet radiation) did not induce apoptosis and only the modulated (not CW) signal was able to induce the effect. The present study did not provide statistically significant support to the hypothesis that RF radiation could enhance apoptosis induced by environmental stress, although caspase-3 activity was consistently higher in the samples exposed to both RF radiation and serum deprivation than in the samples exposed to serum deprivation only. Modulation-specific effects are not supported by the results of the present study.

Conclusions

This study did not provide evidence of effects on ODC activity, proliferation, or caspase-3 activity in L929 cells exposed to 872 MHz RF radiation at 5 W/kg. The results were similar for pulse-modulated and continuous wave radiation. No statistically significant support was found for the hypothesis that altered physiological state (stimulation or stress) would alter cellular responses to RF radiation.

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Chapter 6.

Proliferation, oxidative stress, and cell death in cells exposed to 872 MHz radiofrequency radiation and oxidants

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Abstract

Human SH-SY5Y neuroblastoma and mouse L929 fibroblast cells were exposed to 872 MHz radiofrequency (RF) radiation using continuous waves (CW) or a modulated signal similar to that emitted by GSM mobile phones at a specific absorption rate (SAR) of 5 W/kg in isothermal conditions. To investigate possible combined effects with other agents, menadione was used to induce reactive oxygen species and *tert*-butylhydroperoxide (*t*-BOOH) to induce lipid peroxidation. After 1 or 24 h of exposure, cellular reduced glutathione level, lipid peroxidation, proliferation, caspase-3 activity, DNA fragmentation, and viability were measured. Two statistically significant RF radiation related differences were observed: lipid peroxidation induced by *t*-BOOH was increased in SH-SY5Y (but not in L929) cells, and menadione-induced caspase-3 activity was increased in L929 (but not in SH-SY5Y) cells. Both differences were statistically significant only for the GSM modulated signal. The other end points were not significantly affected in any of the experimental conditions, and no effects were observed from exposure to RF radiation alone. The positive findings may be due to chance, but they might also reflect true modulation-specific effects that occur only in cells sensitized by chemical stress. Further studies are required to investigate reproducibility and dose-response of the possible effects.

Introduction

Rapidly expanding use of mobile communication devices utilizing radiofrequency (RF) radiation has raised public concern about possible health effects from use of such devices. It is well known that absorption of energy from strong RF radiation results in heating of tissues. However, human exposure from mobile communication devices is generally below the level needed for detectable temperature increase, and recent research has focused on possible non-thermal effects from weak RF radiation. It has been suggested that the possible nonthermal effects of RF radiation are linked to amplitude modulation *(1-3)*, although the evidence for modulation-specific effects is not strong *(4)*. Various forms of amplitude modulation are used to carry information in radio communication systems, including digital mobile phones.

Although animal or epidemiological studies do not provide much support to carcinogenic effects *(5, 6)*, several *in vitro* studies have reported effects on cancer-relevant cellular processes such as proliferation, oxidative stress, and apoptosis (programmed cell death) *(7, 8)*. Increased proliferation of cells may lead to uncontrolled growth of transformed cells and thus enhance development of cancer. A number of studies have addressed the effects of RF radiation on cellular proliferation and the results have varied depending on cell type and exposure conditions. Increased proliferation in human glioma cells, lymphocytes and epithelial amnion cells has been reported after exposure to RF radiation *(9-11)*. In contrast, Cleary *et al*. reported decreased proliferation in murine lymphocytes *(12)*, Kwee & Raskmark in human epithelial amnion cells *(13)*, and Capri *et al*. in human lymphocytes *(2)*. Several authors have reported that RF radiation had no significant effects on cellular proliferation in different cell types including also human lymphocytes *(14-22)*.

Apoptosis is characterized by DNA fragmentation, condensation of the chromatin, plasma membrane blebbing, and formation of apoptotic bodies. Improper control of apoptosis causes developmental anomalies and diseases. For example, defects in apoptosis may lead to cell immortalization and tumourigenesis. In recent years, effect of RF radiation exposure on apoptosis has been in interest of many researchers. Although the majority of the studies have indicated no effects on cellular apoptosis *(2, 17, 19, 20, 23-33)*, some reports have suggested increased apoptosis after RF radiation exposure. Peinnequin *et al*. found that Fas-induced apoptosis was enhanced in human Jurkat cells after exposure to RF radiation *(34)* and Maeda *et al*. reported induction of apoptosis in human LoVo cells after microwave treatment *(35)*. Also activation of both p53-dependent and -independent apoptosis pathways in human T-lymphoblastoid leukemia cells has been reported *(36)*. Caraglia *et al*. have suggested that RF induced apoptosis is mediated through the inactivation of the ras/Erk survival signalling due to enhanced degradation of ras and Raf-1 in human epidermoid cancer cells *(37)*. Primary cultures of murine neurons and astrocytes have shown upregulation of apoptosis genes after RF radiation exposure *(38)*. In our previous experiments, RF radiation alone did not affect apoptosis, but enhanced ultraviolet radiation-induced apoptosis in mutant yeast cells *(3)*. This enhancement was observed only in cells exposed to amplitude modulated RF radiation, while no effect was observed in cells exposed to continuous wave (CW) radiation at identical exposure level.

It has been recently suggested that oxidative stress might be the underlying mechanism responsible for the reported cellular effects of RF radiation *(39)*. Most studies have not been able to show effects in different oxidative stress related parameters in mouse macrophages, human monocytes and lymphocytes, human monocytic and erythroid leukemia cells, or murine fibroblasts *(40-44)*. However, one study has shown an increase in cellular reactive oxygen species (ROS) production after combined exposure to RF radiation and iron ions in rat lymphocytes *(45)*.

Differences in the cell lines used, among other methodological differences, might explain the conflicting findings. Also, as suggested by some of the results described above *(3, 45)*, the presumably weak effects of RF radiation might be more easily detectable as an enhancement of the effects of other chemical or physical agents. The aim of the present study was to investigate effects of RF radiation on cellular proliferation, apoptosis, other forms of cell death, and oxidative stress. Two cell lines were used, and the experiments

involved combined exposure with menadione, a chemical agent that affects the endpoints measured through induction of free radicals, and lipid peroxidation inducing agent *tert*-butylhydroperoxide (*t*-BOOH). Proliferation, lipid peroxidation, cellular level of reduced glutathione (GSH), caspase-3 activity, DNA fragmentation, and cell viability (necrotic cell death) were measured.

To assess possible modulation-specific effects, the experiments included exposures to a modulated signal similar to that used in GSM (global system for mobile communications) mobile phones and to a CW (unmodulated) signal. To maximize the probability of finding any effects, a relatively high specific absorption rate of 5 W/kg was used. Various biological effects have been reported *in vitro* at approximately this level *(34, 37, 46, 47)*, and although it is high compared to average human exposure, the safety margin to worst-case local maxima in the head of a mobile phone user is not large *(48)*. The exposure system used in the study allows cooling of the cell cultures to avoid temperature increase that would otherwise result from exposure at 5 W/kg.

Materials and Methods

Reagents

Following reagents were used in cell culture: Dulbecco's modified Eagle medium, Dulbecco's modified Eagle medium with Glutamax I (containing 4.5 g/l glucose), fetal bovine serum (FBS), 50 unit/ml penicillin and 50 µg/ml streptomycin (Gibco, Invitrogen, Carlsbad, CA, USA), and FetalClone II (HyClone, Logan, Utah, USA). In experiments following reagents were utilised: Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA), AlamarBlue reagent (BioSource International, Inc., Camarillo, CA, USA), DEVD-AMC (Calbiochem, CN Biosciences inc., La Jolla, CA, USA), proteinase K (Boehringer Mannheim. Indianapolis, USA) SeaKem GTG agarose (FMC bioproducts, Rockland, Maine, USA), Hepes (Gibco, Invitrogen corp., Paisley, UK), ethylenedinitrilotetraacetic acid Titriplex III (EDTA) (Merck KGaA, Darmstadt, Germany), diphenyl-1-pyrenylphosphine (DPPP), monochlorobimane (mBCL) (Molecular Probes, Leiden, The Netherlands), tryphan blue solution (Sigma-Aldrich CO. LTD, Irvine, UK), DL-dithiothreitol (DTT), ethidium bromide, glycerol, menadione sodium bisulfite, ribonuclease A from bovine pancreas, Triton X-100, Trizma base (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany), *tert*-butylhydroperoxide (Sigma Chemical CO, St. Louis, USA), acetic acid, Nonidet P40 (NP-40) (Roche diagnostics GmbH, Mannheim, Germany).

Cell culture

Two secondary cell lines were exposed to RF radiation. Human neuroblastoma (SH-SY5Y) cells (obtained from Dr. Sven Påhlman, University of Uppsala, Sweden) were cultured in Dulbecco's modified Eagle medium (containing 4.5 g/l glucose) with 10 % heat-inactivated fetal bovine serum (FBS) and 50 unit/ml penicillin and 50 µg/ml streptomycin. Mouse fibroblasts (L929) (purchased from ECACC, European Collection of Cell Cultures, Salisbury, UK) were cultured in Dulbecco's modified Eagle medium with 10 % Fetal Clone II, and 50 unit/ml penicillin and 50 µg/ml streptomycin. The cells were incubated (37 °C, 5 % CO_2) in 75 cm^2 cell culture flasks (Nunc A/S, Roskilde, Denmark). SH-SY5Y cells were harvested by 0.02 % EDTA in PBS and L929 cells by trypsinisation (0.25 % trypsin, 0.02 % EDTA in PBS). Cells were placed in glass (Schott, Mainz, Germany) or plastic (Nunc A/S, Roskilde, Denmark) 55 mm Petri dishes approximately 20 h before each experiment. Plastic Petri dishes were acquired for use after discovering better cell viability in comparison

to glass dishes. Lipid peroxidation and proliferation experiments were performed in glass Petri dishes with both cell lines, as well as caspase-3 activity assay, DNA fragmentation analysis and reduced glutathione assays with L929 cells. Density of the L929 cells were 1.5 x 10^6 except in the viability analysis (2 x 10^6) and controls (0.75 x 10^6) in the caspase-3-like protease activity and DNA fragmentation analyses. SH-SY5Y cells were plated as follows; 2 x 10^6 in caspase-3-like protease activity and DNA fragmentation analysis, 2.5 x 10^6 in reduced glutathione and viability analysis, 3.5 x 10^6 in lipid peroxidation and proliferation studies. Figures for different cell densities in the Petri dishes were obtained from preliminary experiments and were used to optimize each exposure setting.

Exposure protocol

The cells were exposed in four groups: group I, not exposed to RF radiation or chemicals; group II, exposed to RF radiation but not to chemicals; group III, not exposed to RF radiation but treated with a chemical; and group IV, exposed to RF radiation and treated with a chemical. Two different chemicals were used to induce cellular oxidative stress; (1) menadione as a co-exposure chemical in GSH, proliferation, apoptosis, and cell viability studies and (2) *t*-BOOH when studying lipid peroxidation. Preliminary experiments showed that SH-SY5Y cells were more sensitive to chemical exposure than L929 cells and therefore lower doses were used for SH-SY5Y cells. Each dose was chosen to induce such an effect size that it would be possible to observe either a decrease or an increase in the measured parameter. Menadione is a quinone that undergoes one-electron reduction in the mitochondrial respiratory chain, followed by one electron transfer to molecular oxygen, producing $O_2^{-\bullet}$. Oxidative damage caused by *t*-BOOH to cell membranes is considered to be a consequence of membrane-permeant pro-oxidant tert-butoxyl radicals.

The exposures to 872 MHz continuous wave (CW) and GSM modulated (modulation frequency 217 Hz) RF radiation were conducted using a specific absorption rate (SAR) of 5 W/kg. A comprehensive description of the exposure system used with dosimetrical details has been published previously *(49)*. Briefly, the exposure chamber was an aluminium RF-resonator, and the RF power was fed into the chamber with a monopole antenna. Increase of temperature caused by absorption of RF radiation into the cell cultures was eliminated by water circulation, which ensured isothermal conditions (37.0 ± 0.3 °C). Cell culture dishes were placed on a heat exchanger and the temperature of the incoming and outgoing water of the heat exchanger was continuously measured and adjusted by a control unit (Radiation and Nuclear Safety Authority STUK, Helsinki, Finland) with LM45 Precision Centigrade temperature sensors (SOT-23 T4C, order number LM45CIM3, National Semiconductor Corp., Santa Clara, CA, USA). Carbon dioxide (5 % inside the chambers) and temperature-controlled air was provided by ventilation from a cell culture incubator. The exposure system provides a uniform SAR distribution (± 35 % in cell cultures). Two cell dishes were RF-exposed in one chamber and two dishes were simultaneously sham-exposed in an identical chamber. The cells were exposed to the combinations of RF field and the chemicals for 1 h, except for the experiments involving apoptosis (caspase-3 like protease activity and DNA fragmentation analysis), in which exposure time of 24 h was used.

Reduced glutathione (GSH)

After exposure, cells were loaded (15 min, +20 °C, in dark) with final concentration of 100 µM monochlorobimane (MBCL). MBCL forms complex with reduced glutathione and

this can be measured fluorometrically (excitation 380 nm, emission 465 nm, Perkin Elmer HTS 7000 Plus Bio Assay Reader, Norwalk CT, USA). Cells were detached by scraping and suspended to 2.5 ml HBSS buffer, 1.5 ml of cell suspension was transferred to the wells of 48 well plate for determination of GSH and 0.5 ml of suspension was used in protein concentration analysis. Samples for protein concentration analysis were centrifuged (16 000 *g*, 1 min, +4 °C) and supernatant was removed. First, cells were suspended in 100 µl of lysis buffer (10 mM Tris and 1 % Triton X-100 in PBS, pH 7.5) and incubated on ice for 20 minutes at +4 °C. Secondly, samples were centrifuged (13 600 *g*, 45 min, +4 °C) and supernatants were collected in 1.5 ml microcentrifuge tubes. Thirdly, protein concentration was assayed using BioRad Protein Assay Kit following manufacturer's instructions. Results from reduced glutathione analysis were normalized to protein concentration of each sample.

Lipid peroxidation

Lipid peroxidation was examined using a fluorescent probe, diphenyl-1-pyrenylphosphine (DPPP). DPPP integrates to the cell membranes and reacts with lipid hydroperoxides forming a fluorescent DPPP oxide, which can be measured fluorometrically *(50)*. Cells were preloaded before exposure with final concentration of 50 µM DPPP in culture medium (37 °C, 5 % CO_2, 20 min, in dark). After exposure, cells were washed once with 2.5 ml HBSS, scraped and suspended to 2.5 ml HBSS. 1.5 ml of cell suspension was transferred to 48-well plate and levels of DPPP oxide were measured (excitation 340 nm/emission 405 nm, Perkin Elmer HTS 7000 Plus Bio Assay Reader, Norwalk CT, USA). This was followed by protein concentration analysis (0.5 ml of remaining sample). Samples were centrifuged (16 000 *g*, 1 min, +4 °C) and supernatant was removed before protein concentration measurement (performed as in reduced glutathione analyses). Results from the DPPP oxide analyses were normalized to the protein concentration of each sample.

Proliferation

Proliferation was assayed following guidelines in AlamarBlue™ manufacturer's manual. Briefly, 2 x 10^4 SH-SY5Y or 1 x 10^4 L929 cells were plated in 450 µl of medium (48-well plate) and 10 % (50 µl) of AlamarBlue™ was added. This reagent is enzymatically reduced within the cells and the fluorescence is altered, allowing continuous monitoring of the cell number fluorometrically (excitation 540 nm/emission 595 nm, Perkin Elmer HTS 7000 Plus Bio Assay Reader, Norwalk CT, USA). Measurements were made 0, 4, 24, and 48 h after exposure.

Apoptosis

Apoptosis was examined by assessing caspase-3-like protease activity and DNA fragmentation. After exposure, cells were scraped from Petri dishes and centrifuged (310 *g*, 5 min, +4 °C). After this, cells were washed once with 1 ml PBS and supernatant was removed. This was followed by protein concentration analyses (as in reduced glutathione analysis). After protein concentration analyses, remaining samples were diluted to contain 1 mg protein/ml with assay buffer (20 mM Hepes, 10 % glycerol, 2 mM DTT, pH 7.5). After this, 50 µl fluorogenic 120 µM caspase-3 substrate, DEVD-7-amino-4-methylcoumarin (AMC), 50 µl diluted samples (50 mg protein) and 200 µl assay buffer were transferred in to the wells of 96 well plate and incubated in a cell culture incubator for 1 hour. Activity of caspase-3-like proteases cleaves fluorogenic caspase-3 substrate and liberates AMC which fluorescence can be measured fluorometrically (excitation 380 nm, emission 465 nm, Perkin Elmer HTS 7000 Plus Bio Assay Reader, Norwalk CT, USA).

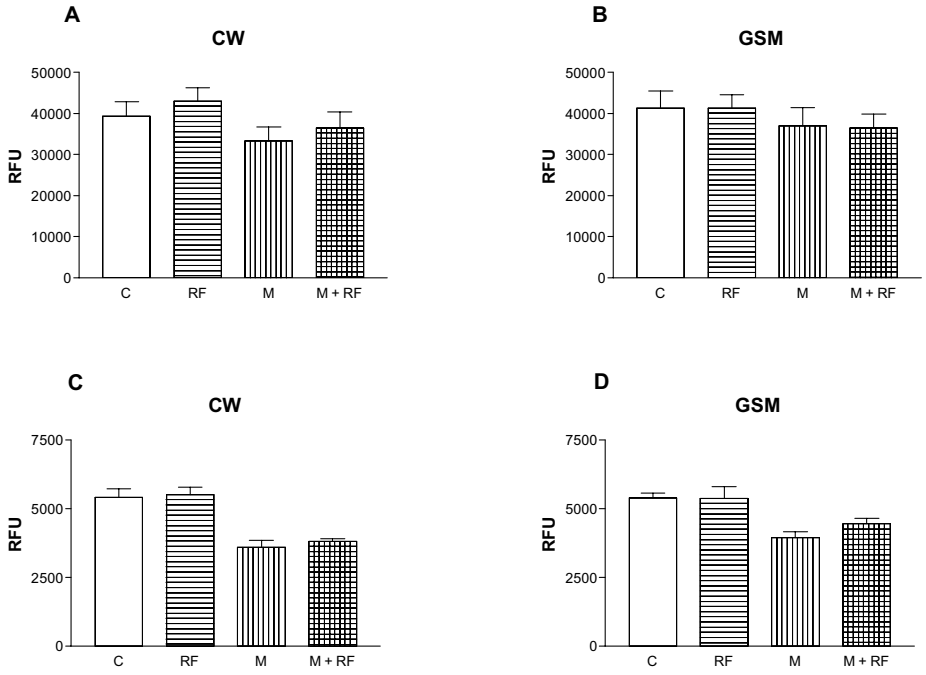

Figure 1. Reduced glutathione levels in L929 fibroblasts (A-B) and SH-SY5Y neuroblastoma cells (C-D) exposed to continuous wave (CW) or GSM-modulated RF radiation at 5 W/kg. C = control, RF = exposed to RF radiation, M = menadione (A-B: 100 µM, C-D: 20 µM) treated cells, M + RF = menadione treated cells exposed to RF radiation. RFU = relative fluorescence units. Menadione decreased significantly ($p \leq 0.001$) reduced GSH levels in both cell lines. The data are represented as mean ± SEM.

Internucleosomal DNA fragmentation analysis was used for verifying the result obtained from caspase-3-like protease activity assay. DNA fragmentation analysis is based on fragmentation of DNA to 180-185 base-pair fragments during apoptosis and this can be determined by using agarose gel electrophoresis. After exposure, the cells were scraped in culture medium and centrifuged (310 *g*, 5 min, +4 °C), supernatant was removed and samples were frozen (-70 °C). Before analysis, samples were thawed at +4 °C and lysed with 80 µl of lysis buffer (TAE; 40 mM Tris, 0.11 % acetic acid, 1 mM EDTA, 0.25 % NP-40). This was followed by incubation on ice for 30 minutes and centrifugation (16 000 *g*, 30 min, +4 °C). After centrifugation, 70 µl of supernatants were transferred in new microcentrifuge tubes and 8 µl of 10 mg/ml RNAase A was added and samples were incubated for 1 h, at +50 °C. This was followed by addition of 9 µl of 10 mg/ml proteinase K and further incubation for 1 h at +50 °C. Samples were electrophoresed on 1.5 % agarose gel at 100 V for 1 h and stained with 0.5 µg/ml of ethidium bromide. Agarose gels were analyzed and photographed with BIO-RAD Gel doc 2000 (Milan, Italy) and Quantity One (v. 4.6.3) program.

Cell viability

Cell viability was determined using 0.4 % tryphan blue dye. Samples and tryphan blue were mixed in 1:1 ratio. Tryphan blue enters dead cells via damaged cell membrane and

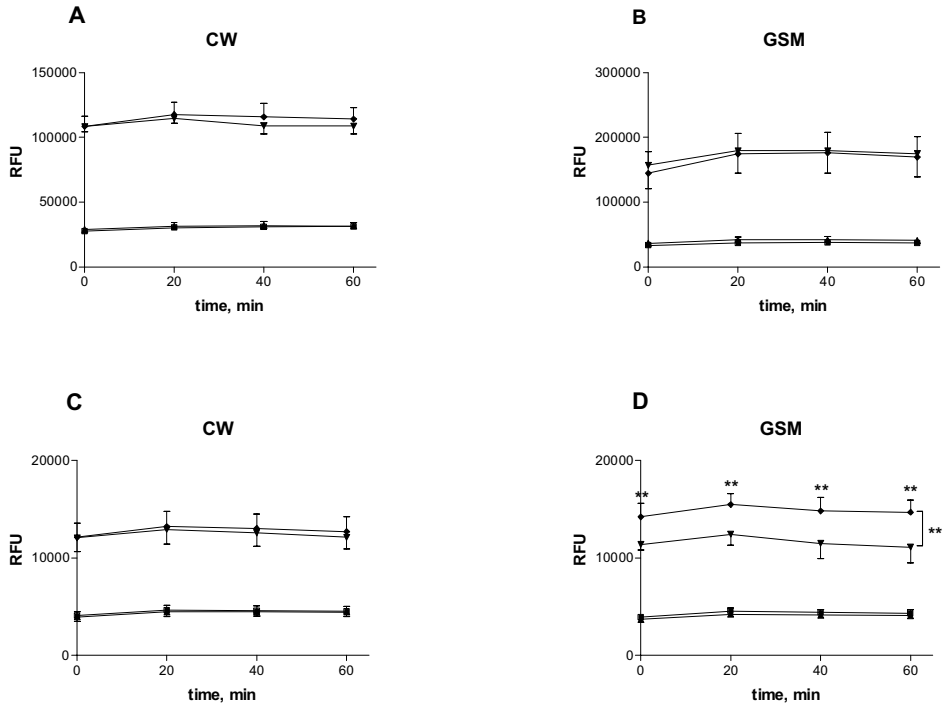

Figure 2. Lipid peroxidation levels in L929 fibroblasts (A-B) and SH-SY5Y neuroblastoma cells (C-D) exposed to continuous wave (CW) or GSM-modulated RF radiation at 5 W/kg. ■= control, ▲= exposed to RF radiation, ▼= *t*-BOOH (A-D: 0.5 mM) treated cells, ♦= *t*-BOOH treated cells exposed to RF radiation. RFU = relative fluorescence units. Addition of *t*-BOOH resulted in significantly ($p \leq 0.001$) increased lipid peroxidation in both cell lines. The data are represented as mean ± SEM.

intact (living cells) remain to be uncolored. This change was observed by phase contrast light microscope (Olympus CK40-F200 & CK2-TR, Japan) and counted as proportion of living cells to the number of all (dead+living) cells using Bürger cell counting chamber (Marienfeld, Germany).

Statistical analysis

Statistical analyses using raw values were performed using SPSS for Windows release 14.0.1 (SPSS Inc., Chicago, Illinois, USA). A linear mixed model analysis was performed in which replicate (3–5 independent replications were performed for each end point and modulation type investigated) was included as a random factor and exposure to RF radiation, exposure to menadione or *t*-BOOH, signal type (CW or GSM), possible measurement time, and their interactions, were studied as fixed effects. An additional random effect, dish, was included in analyses where multiplemeasurements from the same dish were performed in the course of time. Pairwise comparisons between RF-exposed and non-RF-exposed samples were performed as post tests. A p value < 0.05 was considered statistically significant.

Results

Reduced glutathione (GSH)

Addition of menadione (100 µM for L929, 20 µM for SH-SY5Y) resulted in significantly (p

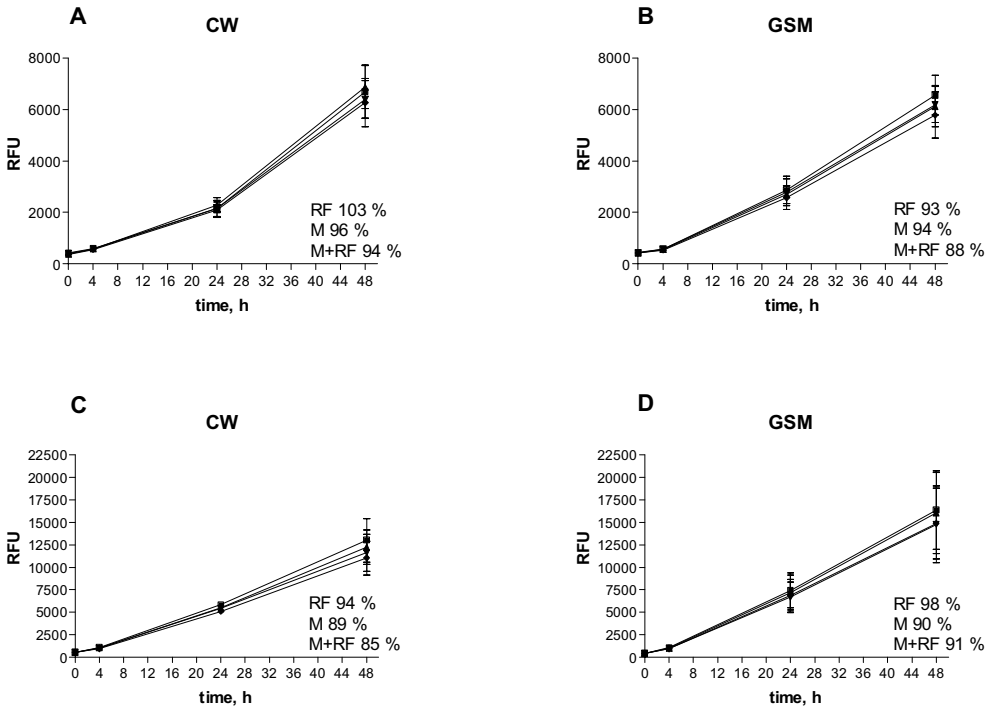

Figure 3. Cell number (AlamarBlue fluorescence) in L929 fibroblasts (A-B) and SH-SY5Y neuroblastomas (C-D) exposed to continuous wave (CW) or GSM-modulated RF radiation at W/kg. ■= control, ▲= exposed to RF radiation, ▼=menadione (M) treated cells (A-D: 10 µM), ♦= menadione treated cells exposed to RF radiation. RFU = relative fluorescence units. Percentages given on figures are the relative AlamarBlue fluorescence of the exposed sample to control at 48 h time point. The data are represented as mean ± SEM.

≤ 0.001) reduced GSH levels in both cell lines (Figure 1). After RF radiation exposure, GSH levels were not generally affected. In L929 cells the overall test indicated a significant (p = 0.013) RF radiation-related increase, but post tests did not show significant differences in GSH levels between RF-exposed and control cells or between the menadione-exposed and menadione+RF-exposed cells.

Lipid peroxidation

t-BOOH (0.5 mM) increased lipid peroxidation significantly ($p \leq 0.001$) in both cell lines (Figure 2). A further significant (p = 0.002) increase in lipid peroxidation was observed in SH-SY5Y cells exposed to GSM-modulated RF radiation. The increase was statistically significant at all time points (0 min: 25 %, p = 0.005, 20 min: 24 %, p = 0.004, 40 min: 29 %, p = 0.002, and 60 min: 32 %, p = 0.001). Lipid peroxidation in SH-SY5Y cells was not affected by the CW signal. No RF radiation-related differences in L929 cells were detected.

Proliferation

Exposure to menadione (10 µM) slightly decreased cellular proliferation in both cell lines (Figure 3). No statistically significant effects of RF radiation on cellular proliferation were observed.

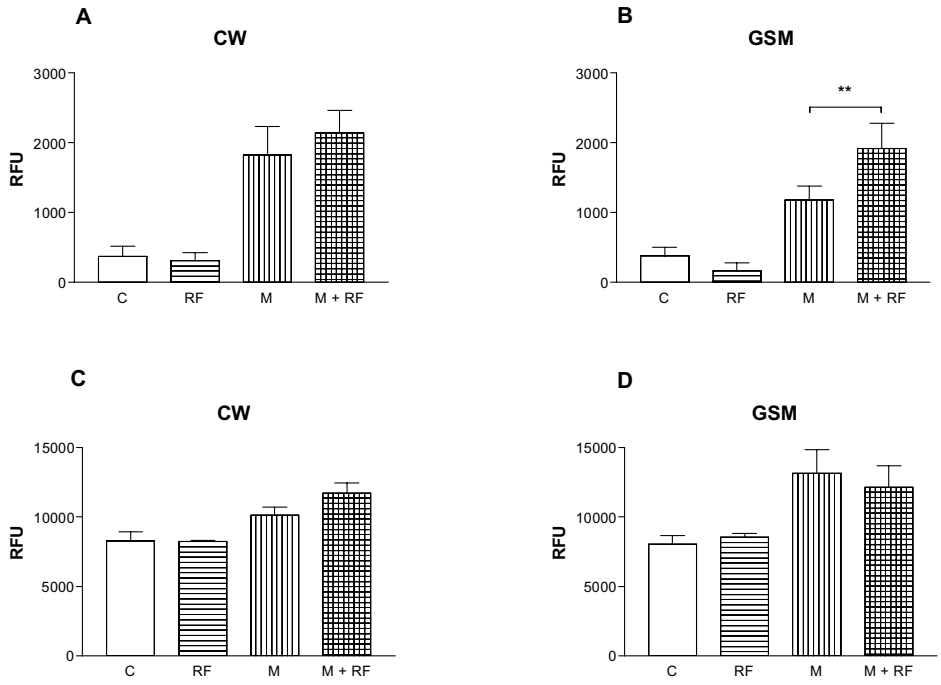

Figure 4. Caspase-3-like protease activity in L929 fibroblasts (A-B) and SH-SY5Y neuroblastomas (C-D) exposed to continuous wave (CW) or GSM-modulated RF radiation at 5 W/kg. C = control, RF = exposed to RF radiation, M = menadione (A-B: 50 µM, C-D: 10 µM) treated cells M + RF = menadione treated cells exposed to RF radiation. RFU = relative fluorescence units. Exposure to menadione resulted in significantly ($p \leq 0.001$) increased caspase-3 activity in both cell lines. The data are represented as mean ± SEM.

Caspase-3 activity

Exposure to menadione (50 µM for L929, 10 µM for SH-SY5Y) resulted in significantly ($p \leq 0.001$) increased caspase-3 activity in both cell lines (Figure 4). In L929 cells stimulated with menadione, cellular caspase-3 activity was significantly ($p = 0.008$) higher in the RF radiation exposed cells than in those exposed to menadione only. In post tests, the increase (17 %) associated with the CW signal was not statistically significant, but the increase (63%) associated with GSM modulated RF radiation was statistically significant at $p = 0.008$.

DNA ladder

In SH-SY5Y cells exposure to menadione (50 µM for L929, 10 µM for SH-SY5Y) caused clear DNA ladder pattern in agarose gel and also in control and RF only exposed samples a very faint ladder was seen. RF radiation had no effect on DNAfragmentation in SH-SY5Y cells. In L929 cells no detectable DNA fragmentation was observed in any of the exposure groups.

Cell viability

Exposure to menadione (100 µM for L929, 20 µM for SH-SY5Y) decreased viability of both cell lines and the decrease was significant ($p \leq 0.001$) in SH-SY5Y cells, but RF radiation

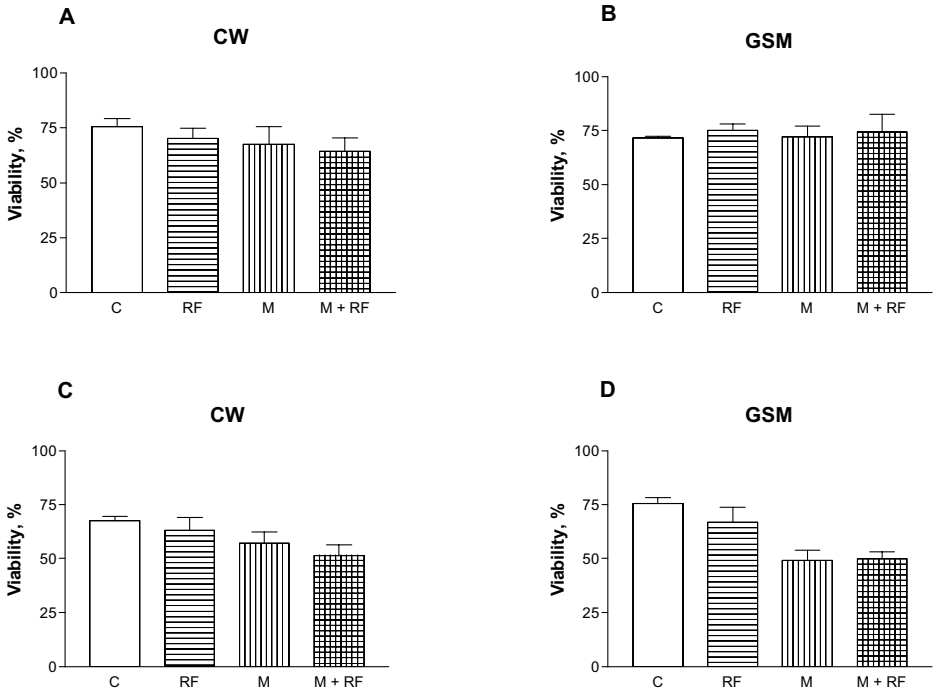

Figure 5. Cell viability in L929 fibroblasts (A-B) and SH-SY5Y neuroblastomas (C-D) exposed to continuous wave (CW) or GSM-modulated RF radiation at 5 W/kg. C = control, RF = exposed to RF radiation, M = menadione (A-B: 50 μM, C-D: 10 μM) treated cells M + RF = menadione treated cells exposed to RF radiation. Exposure to menadione decreased viability significantly ($p \leq 0.001$) in SH-SY5Y cells. The data are represented as mean ± SEM.

did not significantly affect viability (Figure 5).

Discussion

The only RF radiation related statistically significant differences in the present study were an increase in chemically induced lipid peroxidation in SH-SY5Y cells and an increase in caspase-3-like protease activity in L929 cells. The increase in lipid peroxidation in SH-SY5Y cells was associated only with GSM modulated RF radiation. As the increase was not supported by a similar finding in the other cell line used, further studies are needed to determine whether this is a chance finding or reflects a true modulation-specific biological effect in this cell line. To our knowledge, the present study is the first that has addressed effects of RF radiation on lipid peroxidation *in vitro*, which underlines the need to investigate the reproducibility of the finding. Similarly with the lipid peroxidation findings, the RF radiation related increase in caspase-3-like protease activity was observed only in one cell line and only in the cells exposed to the GSM modulated radiation. In this case, however, there was also a small, not statistically significant increase in the cells exposed to the CW signal. There are thus three possible interpretations: 1) a modulation specific effect, 2) a true effect, which was (by chance) not statistically significant for the CW signal and 3) a false positive finding in the GSM-exposed cells. The majority of previous studies *(2, 17, 19,*

20, 23-33) have not shown any effects of RF radiation on cellular apoptosis. However, as some previous studies have indicated possible apoptosis-enhancing effects of RF radiation *(3, 34-38),* further studies are warranted to explore the reproducibility and dose-response of the possible effect.

No effects of RF radiation on cellular glutathione levels were found in either of the cell lines. This indicates that no persistent oxidative stress during RF radiation exposure was induced, although we cannot exclude possible short-term variations in cellular level of reactive oxygen species. This finding is concordant with the majority of previous studies reporting no significant effects on oxidative stress from RF radiation exposure *(40-44)*. The lack of effects on glutathione level does not support the hypothesis that oxidative stress would explain the statistically significant findings observed in the present study. The finding of increased lipid peroxidation is difficult to explain without effects on oxidative stress. However, the increase in caspase-3-like protease activity could be a true biological effect even without effects on oxidative stress, as apoptosis is known to be regulated by multiple cellular pathways *(51)*.

In the present study, caspase-3-like protease activity was validated by DNA fragmentation assay. Results from DNA fragmentation analyses confirmed menadione-induced apoptosis in SH-SY5Y cells. However, DNA fragmentation was not seen in L929 cells. This is consistent with other studies that have reported problems in DNA fragmentation analyses with L929 cells *(52)*. The lack of confirmation by the fragmentation assay complicates the interpretation of the positive caspase-3 findings in L929. However, a change in caspase-3-like protease activity may serve as an indicator of a biological response even if we cannot confirm that this change reflects an effect on apoptosis.

Proliferation of the cells was not significantly affected by exposure to RF radiation. This result is consistent with the majority of the previous studies, which have generally reported no effects on proliferation from RF radiation *(14-21)*. According to our results, RF radiation has no effects on cell viability (necrotic cell death), which is also consistent with previous studies *(27, 40)*.

Conclusions

Two statistically significant findings of this study suggest that RF radiation might enhance chemically induced lipid peroxidation and caspase-3 activity. Both suggestive effects were seen in only one of the two cell lines tested, the responding cell lines were different for the two effects, and only the GSM modulated signal produced statistically significant differences. As a relatively high exposure level (5 W/kg) was used in this study, additional experiments would be needed to investigate reproducibility and dose-response of the positive findings. The results do not support induction or enhancement of oxidative stress in cells exposed to RF radiation, as cellular GSH levels were not affected. Proliferation and cell viability were not affected in any of the experimental conditions. RF radiation alone, without stress-inducing chemical agents, had no effects on any of the endpoints measured.

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Chapter 7.

General discussion

1 Proliferation and cell death related effects of RF radiation on cell cultures

1.1 Overview

The present work indicated that RF radiation may interfere with cellular ODC activity in rat primary astrocytes. Some effects on cellular apoptosis and lipid peroxidation in secondary cell lines were also detected, but the significance of those observations is unclear, as the changes detected were small and inconsistent (for a summary of the results see Table 1).

It is noteworthy that *in vitro* RF radiation studies suffer from their own unique limitations, which have to be taken into account when interpreting the results. For example, different exposure set-ups and their temperature control systems as well as choice and handling of cells may affect the study results significantly. In the present study, the exposures were arranged in a TEM cell set-up and a waveguide set-up. A major effort was made to characterize both set-ups adequately, so that SAR distribution and heating effect of the RF radiation in the cell culture dishes were characterised.

The results of the present study emphasise the importance of temperature control in RF radiation studies using cell cultures. The studies presented in Chapter 2 clearly showed that cellular ODC activity was sensitive to small temperature differences in the exposure chambers, and the experiments described in Chapter 3 indicated that differences in cooling methods can affect the results obtained with different exposure systems.

The study of the possible non-thermal effects of RF radiation is challenging as non-thermal effects of RF radiation can be studied only at relatively low exposure levels. At exposure levels producing notable heating, some kind of temperature compensation has to be used to prevent the effects of temperature elevation. The two exposure systems used in the present study both had cooling systems for the cell cultures to enable exposures at quite high (up to 6 W/kg) SAR levels. These higher SAR levels were included in the study to maximise the probability of finding non-thermal effects of RF radiation.

The present work did not confirm the hypothesis that the physiological condition of cells (normal/activated/stressed) would modify cellular responses to RF radiation and possibly explain the discrepancies in the results of previous studies. Some indications were found to support the hypothesis that chemical stressors may sensitise cells to RF radiation, though more studies will be needed to clarify this issue.

1.2 ODC and proliferation

In the present study, ODC activity was decreased in rat primary astrocytes after exposure to CW and GSM signals at SAR levels of 1.5 and 6.0 W/kg with a waveguide set-up (Chapter 4). This is an interesting finding because the response was consistent and it was seen at both exposure levels and signals. No indications of any modulation specific responses were detected in contrast to some previous studies (Litovitz *et al.* 1993&1997, Penafiel *et al.* 1997). This result also indicates that the biological effects of RF radiation may be dependent on cell type. No consistent effects on ODC activity were observed using secondary cell lines. For example, in one study (Chapter 3) ODC activity was increased in L929 cells after exposure to DAMPS modulated signal in

Table 1. Main findings of the present study. Exposures at 835 MHz were carried out in a TEM cell exposure set-up and other exposures at 872 and 900 MHz in a waveguide set-up.

End point measured	Cell line	RF radiation exposure	Other exposure	RF radiation effects	Chapter
ODC activity		**835 MHz CW/DAMPS**			
	L929	SAR 2.5, 6.0 W/kg, 2, 8, 24 h	-	**Decreased** after 2 h 6.0 W/kg CW & DAMPS exposure (temperature)	3
ODC activity		**872 MHz CW/DAMPS**			
	L929	SAR 6.0 W/kg, 8 h	-	**Increased** after DAMPS exposure	3
ODC activity		**900 MHz CW/GSM**			
	L929	SAR 0.2, 0.4 W/kg, 2, 8, 24 h	-	No effects	2
ODC activity		**872 MHz CW/GSM**			
	L929	SAR 1.5, 2.5, 6.0 W/kg; 2, 8, 24 h	-	**Decreased** after 24 h exposure to 1.5 W/kg GSM and 2 h exposure to 6.0 W/kg CW (temperature)	4
	SH-SY5Y	SAR 1.5, 2.5, 6.0 W/kg; 2, 8, 24 h	-	**Decreased** after 2 h exposure to 6.0 W/kg CW	4
	C6	SAR 1.5, 2.5, 6.0 W/kg; 2, 8, 24 h	-	No effects	4
	Rat primary astrocytes	SAR 1.5, 6.0 W/kg; 2, 8, 24 h	-	**Decreased** in 11/12 experiments (CW & GSM)	4
	L929	SAR 5.0 W/kg, 1, 24 h	-	**Increased** after 1 h GSM exposure	5
			Fresh medium	No effects	
	L929	SAR 5.0 W/kg 1 h	-	No effects	5
			Serum deprivation	No effects	
GSH levels	L929	SAR 5.0 W/kg 1 h	-	No effects	6
			Menadione	No effects	
	SH-SY5Y	SAR 5.0 W/kg 1 h	-	No effects	
			Menadione	No effects	
Lipid peroxidation	L929	SAR 5.0 W/kg 1 h	-	No effects	6
			t-BOOH	No effects	
	SH-SY5Y	SAR 5.0 W/kg 1 h	-	No effects	
			t-BOOH	**Increased** after GSM exposure	

Table 1. Continued.

End point measured	Cell line	RF radiation exposure	Other exposure	RF radiation effects	Chapter
Proliferation	L929	SAR 5.0 W/kg 24 h	- Fresh medium	No effects No effects	5
	L929	SAR 5.0 W/kg 1 h	- Menadione	No effects No effects	6
	SH-SY5Y	SAR 5.0 W/kg 1 h	- Menadione	No effects No effects	
Caspase-3 activity	L929	SAR 5.0 W/kg 1 h	- Serum deprivation	No effects No effects	5
			- Menadione	No effects **Increased** after GSM exposure	6
	SH-SY5Y		- Menadione	No effects No effects	
DNA ladder	L929	SAR 5.0 W/kg 1 h	- Menadione	No effects No effects	6
	SH-SY5Y		- Menadione	No effects No effects	
Viability	L929	SAR 5.0 W/kg 1 h	- Menadione	No effects No effects	6
	SH-SY5Y		- Menadione	No effects No effects	

the waveguide set-up, but no other significant effects on those cells were detected after exposing them in the same set-up.

Primary cells have been seldom used in RF radiation studies. Only two reports were found in the literature which have examined the effects of RF radiation on primary astrocytes. Lee *et al.* (2006) reported no effects on the cellular stress response measured as expression of heat shock proteins and activation of mitogen-activated protein kinases after exposure to 1763 MHz radiation at 2 or 20 W/kg for 30 min or 1 h. The results were similar using human T-lymphocytes, pointing to no differences between those two cell types. Zhao *et al.* (2007) exposed mouse primary neurons and astrocytes to 1900 MHz radiation emitted by a GSM mobile phone and reported upregulation of caspase-2, caspase-6, Bax, and Asc (apoptosis associated speck-like protein containing a card) gene expression. In neurons, gene expression was affected with both the "stand-by" and "on" modes of the mobile phone, but in astrocytes only with the "on" mode. The reliability of findings of Zhao et al. (2007) is severely limited by the use of a poorly controlled exposure set-up (mobile phone situated above the culture dishes), which complicates the interpretation of the results.

The studies of Lee *et al.* (2006) and Zhao *et al.* (2007) did not compare primary astrocytes to secondary cell lines. Therefore, more studies are warranted to clarify the possible sensitivity of primary astrocytes to RF radiation.

In the present study, proliferation of L929 and SH-SY5Y cells was not significantly altered by RF radiation exposure (Chapters 5 and 6). This supports our findings on ODC activity since no clear and reproducible effects on cellular ODC activity in those cell lines were detected.

1.3 Oxidative stress

Cellular GSH levels in L929 and SH-SY5Y cells were measured in the present study as an indicator of possible long-term oxidative stress after RF radiation exposure. Since no significant effects on cellular GSH levels were detected, it seems probable that no long-term oxidative stress had been induced in the exposed cells (Chapter 6). This confirms the findings of Zeni *et al.* (2007), who were unable to detect any RF radiation induced changes in cellular ROS production in L929 cells. In the present study, however, RF radiation was observed to modulate chemically induced lipid peroxidation in SH-SY5Y cells (Chapter 6). The increase was observed only in one of the two cell lines exposed to one of two signals tested and therefore the finding needs independent confirmation. However, since lipid peroxidation is a chain reaction which can be initiated by small, transient increase in ROS production, it is theoretically possible that RF radiation can evoke lipid peroxidation without affecting cellular GSH levels. There are no other reports on the effects of RF radiation on lipid peroxidation in mammalian cells. Tkalec *et al.* (2007) reported that 900 MHz RF radiation at field strengths of 10–120 V/m for 2 h increased lipid peroxidation in duckweed *Lemna minor* L.

1.4 Cell death

In the present study, apoptosis measured as caspase-3 activity was increased in L929 fibroblasts after combined exposure to menadione and GSM modulated 872 MHz signal at 5 W/kg (Chapter 6). A similar (but not significant) increase was also detected in cells exposed to combined treatment with menadione and the CW signal. It is therefore difficult to elucidate whether there is any true difference between the unmodulated and GSM-modulated signals. It is of interest to

compare all caspase-3 results from experiments that combined RF exposure with other stressors. Figure 2 of Chapter 5 shows that caspase-3 activity was non-significantly higher in L929 cells after combined exposure to RF radiation and serum deprivation than in cells exposed to serum deprivation only. The finding is similar for CW and GSM signals and also resembles the L929 findings from the experiments where RF radiation was combined with menadione (Figure 4 of Chapter 6). It would be interesting to perform further experiments to find out whether this apparently consistent pattern of results is due to chance or reflects a true, even though weak, RF effect on stressed L929 cells, which reached statistical significance only in one case.

Cellular viability was not affected after RF radiation exposure in the present study (Chapter 6). This supports the findings of all previous studies presenting the results of viability assays, since no evidence of cell lethality has been reported.

1.5 Modulation specific responses

The majority of data produced in the present study did not support the hypothesis that there are modulation specific responses in cell cultures. The decreased ODC activity in rat primary astrocytes, which was the clearest biological effect observed, was similar for unmodulated and modulated signals. Suggestive evidence was found for a modulation specific response in lipid peroxidation, since this was increased only in cells exposed to GSM modulated RF radiation. However, this finding needs to be independently confirmed.

2 Conclusions

In the present study, ODC activity in secondary cell lines was not affected by RF radiation, irrespective of the signal characteristics and exposure levels used. There were no differences between the two exposure chamber types (TEM cell and waveguide) used and furthermore there were no consistent effects on ODC activity in secondary cell lines exposed in either of the exposure chambers. However, ODC activity was affected consistently in several experiments in rat primary astrocytes exposed to RF radiation isothermally at 1.5 or 6 W/kg. This indicates that the effects of RF radiation may be cell type specific and that more studies are warranted with primary cells.

Stressed cells were generally not more sensitive to RF radiation than normal cells. There was some evidence that RF radiation could induce an increase in caspase-3 activity and lipid peroxidation in cells treated with stress inducing agents, but these findings will require independent replication, as statistically significant changes were reached only in individual cases. No effects on cellular GSH levels, proliferation, or viability were detected in stressed or normal cells.

The results do not support the existence of modulation-specific effects: when any effects of RF radiation were observed, in general there were no differences in the responses to modulated and CW signals.

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