EFFECT ON DNA REPAIR GENES TRANSCRIPTION IN CULTURED CELLS OF 2.45 GHZ MICROWAVE EXPOSURE.

A. Perrin[(1)], C. Bachelet[(2)], C. Fournier[(3)], P. Levêque[(4)], A. Collin[(5)] and A. Peinnequin[(6)]

[(1)]Molecular and Cellular Biophysics Unit of the Health Service Research Center for Defense (CRSSA), BP 87, 38702 La Tronche cedex, France. Email : aperrin@crssa.net

[(2)]as (1) above, but Email : cbachelet@crssa.net

[(3)]Radiobiology and Inflamation Unit of the Health Service Research Center for Defense (CRSSA), BP 87, 38702 La Tronche cedex, France. Email : cfournier@crssa.net

[(4)]Research Institut on Microwave and Optical Communications (IRCOM), CNRS UMR 6615, 87060 Limoges, France. Email : leveque@unilim.fr

[(5)]As (4) above, but Email : alice.collin@unilim.fr

[(6)]As (3) above, but Email : apeinnequin@crssa.net

ABSTRACT

The aim of the study is to investigate, *in vitro*, the effect of 2.45 GHz continuous (CW) and pulsed (PW) electromagnetic field exposure combined with a known mutagen on the induction of enzymes implicated in the DNA repair pathway. Microwaves do not create bonds breaks within molecules and there is no clear hypothesis for a possible mechanism supporting a biological action. Nevertheless, an indirect influence of microwaves during an intermediary step of the complex sequence of events involved in mutagenesis cannot yet be excluded.

Highly sensitive real-time RTqPCR was used to monitor transcriptional variations of DNA repair genes. The experiments were carried out using the monocyte human cell line THP1 with the genotoxic compound 4-nitroquinoline-N-oxide (4-NQO).

The carrier frequency was 2.45 GHz CW and PW (1 kHz repetition time, 10 % duty cycle) with the same power density corresponding to an average SAR value of 0.19 W/kg in the biological samples. Non exposed (sham) and exposed (PW and CW) cell culture plates (N=6 for each condition) were incubated simultaneously in three identical incubators in the presence of 4-NQO, under shaking, at 37°C. Specially designed incubators were integrated in three identical anechoic chambers equipped with waveguide antennas. Care was taken to increase the reproducibility of the experiments and to avoid false positive or misinterpretation of the results. The presence or the absence of the electromagnetic field was the only difference between the sham and exposed assays. The different exposure conditions were applied alternatively in the three anechoics chambers in order to avoid cage effects.

The temperature inside the cell plates was measured with an optic fiber probe (Luxtron). Numerical dosimetry was calculated using the Finite Difference Time Domain method. A time-scaled form of the heat transfer equation allowed to calculate the temperature distribution inside the petri dishes. Interactions between the electromagnetic fields and the Plexiglas multi-blade structure of the incubator have to be considered. The SAR distribution and the temperature depends on the petri dishes positions. The exposure power level was controlled and didn't induce temperature increases in the cell culture medium.

First, the genes implicated in DNA repair after 4-NQO treatment were screened using thematic human DNA repair GEArray™ (SuperArray). Four targets dramatically modified at the transcriptional level were chosen : APEX, RAD 54, RAD52, MSH6. The expression of these genes normalised using geometric average of three internal control genes (PPIA, ACTB and HPRT) was assessed using a LightCycler real-time PCR device. Each experiment was reproducibly repeated 3 times.

In our experimental conditions, after the RT-PCR quantification of the target genes expression, no effect of CW and PW 2.45 GHz microwave was observed on the reactivity of the cell DNA repair system induced by the treatment with a known mutagenic agent.

Research supported by the state organization supervising the manufacture and sale of armaments (DGA).

INRODUCTION

An effect of microwaves in cancer development has never be clearly and reproducibly shown [1] but an indirect influence during an intermediary step of the complex sequence of events involved in mutagenesis cannot yet be excluded. Real-time RT-PCR has now become one of the most widely used methods of gene quantification because, with a well-designed experiment performed with the proper controls, it is a highly sensitive, efficient, fast and reproducible method for measuring gene expression [2].
Using this technique, the aim of the study was to investigate, *in vitro*, the effect of 2.45 GHz continuous (CW) and pulsed (PW) electromagnetic field exposure combined with a known mutagen on the induction pattern of enzymes implicated in the DNA repair [3]. The average SAR inside the dishes was 0.19 W/kg and the pulse sequence was commonly used for classical radars. The experiments were carried out using the human monocyte cell line THP1 with the chemical mutagen 4-nitroquinoline-N-oxide (4-NQO).
In a first step, the genes of interest have been identified in order to further study the effect of microwave exposure on the reactivity of the cells to a mutagen agent. These targets have to be highly expressed and to present a regular kinetic in response to the mutagen treatment favoring an easier observation of any modification (delay, increase…) in the expression pattern. A great number of genes implicated in DNA repair after 4-NQO treatment were screened using thematic human DNA repair GEArrayTM (SuperArray). In a second step, real-time RTqPCR was used to monitor transcriptional variations of DNA repair genes.

MATERIALS AND METHODS

Exposure set-up and dosimetry

Specially designed thermo regulated Plexiglas incubators were installed in three identical anechoic and submitted to rotation by an external mechanical system. The temperature of the cell cultures was maintained by water circulation and controlled by a cryostat.
The signal source was composed of a high frequency 2.45 GHz magnetron generator (Sairem, France), a signal modulator (Agilent, France), a horn antenna in a vertical position (Celti, France). The field was measured by a wide band fieldmeter (EMtest, France). The system was controlled by computer with a specifically designed program (Labview) allowing the parallel recording of the chamber temperature and the average power values during each experiments . The carrier frequency was 2.45 GHz CW and PW (1 kHz repetition time, 10 % duty cycle) with the same average power density.
For dosimetry analysis, the temperature inside the cell plates was measured with fluoro-optic fiber probes (Luxtron). Numerical dosimetry was calculated using the Finite Difference Time Domain taking into account the design of the setup to model it, the petri dishes location, the exposure parameters and the biological parameters [4].

Cell culture

The THP1 cells were grown, in suspension ($1.5x10^6$ cells/ml), in 35 mm petri dishes containing 1.5 ml of RPMI 1640 culture medium + glutamax with 10%SVF and 25 mM Hepes. After incubation in the experimental conditions, the cells (10^6/sample) were washed (PBS) and centrifuged. The pellet was resuspended in lysis buffer and kept at –80°C until blind analysis.

Macroarrays

Total RNA was isolated from 10^6 cells using RNeasy mini kit (QIAGEN, Courtabeuf, France) with optional DNAse treatment according to the manufacturer's instructions. Radioactive probes were synthesized from 1 µg RNA using AmpoLabeling-LPR Kit (SuperArray, Frederick, USA) and [32P]-dCTP (3,000 Ci/mmol, Amersham, Les Ullis, France) according to the manufacturer's instructions. Probes were hybridized on GEArray Q Series human DNA Damage Signaling Pathway gene array kit (SuperArray, Frederick, USA) according to the manufacturer's instructions. Data were acquired with a Storm 840 and analyzed using ImageQuant software (Amersham, Les Ullis, France)

mRNA extraction and Reverse Transcription

Messenger RNA was isolated from the cells using MagnaPure LC mRNA Isolation Kit I (Roche Applied Science, Mannheim, Germany) in a MagnaPure LC instrument according to manufacturer's instructions. mRNA was eluated in a 50µL final volume. Reverse transcription was carried out in a 10µL final volume of mRNA-eluate (8.10^4 cells) using

MMLV-based Reverse Transcriptase Core kit (Eurogentec, Saraing, Belgium) using oligo (dT)15 as primer, according to the manufacturer's instructions.

Primers design

Oligonucleotide primers were synthesized by Eurogentec (Saraing, Belgium). Primer design was done with MacVector software (Accelrys, San Diego, USA) to obtain short (80-130 nt) intron-spaning amplicons. Specificities of the PCR amplification were documented with LightCycler melting curve analysis. Melting peaks, obtained either from RT-product or from specific recombinant DNA, were identical.

Real-Time Quantitative RT-PCR

The PCR was carried out with the LC Fast Start DNA Master SYBR Green kit (Roche Applied Science, Mannheim, Germany) using 0.25µL of cDNA (≠200 cells) in a 20µl final volume, 4mM MgCl2 and 0.4µM of each primer (final concentration). Quantitative PCR was performed using a Lightcycler (Roche Applied Science, Mannheim, Germany). The crossing point (CP) value, corresponding to the PCR cycle number at which fluorescence was detected above threshold, was calculated with the Lightcycler Software v.3.5 (Roche Applied Science, Mannheim, Germany) using the second derivative maximum method. Quantification was achieved using a pool of cDNA samples as calibrator according to the comparative threshold cycle method [5]. Relative mRNA values were calculated using geometrical average [6] of three reference genes, i.e. CyclophilinA, HPRT and beta-Actin, with RealQuant software (Roche Applied Science, Mannheim, Germany).

RESULTS

For biological experiments, an average SAR value inside the dishes of 0.19 W/kg was determined by numerical calculation . The variation of the temperature of the medium was less than 0.4 ± 0.1 °C after 2 h of electromagnetic field exposure (far field) measured in 37°C pre-equilibrated culture medium in the Petri dishes.
For preliminary screening, a commercial gene array kit designed to profile the expression of 96 key genes involved in DNA damage signaling pathways was used. The cells were incubated in the absence (control) or in the presence of 4-NQO for 2 h. After treatment, the medium was eliminated and the cells were allowed to grow in mutagen free culture medium for 1 and 2 hours recovery (Fig.1).
Four targets were dramatically modified at the transcriptional level as a function of time : APEX, ATRX, RAD52, MSH6. These genes are coding for the APEX nuclease stimulating the DNA binding of several transcription factors, the chromatin-remodeling protein ATRX, RAD52 involved in homologous recombination and a replication mismatch repair protein MSH6.

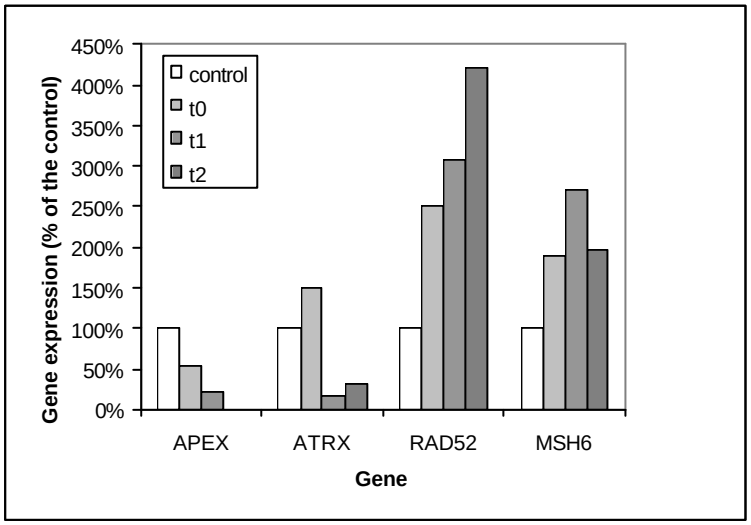

Fig.1. Expression of the target genes 0 (t0), one (t1) and two (t2) hours after treatment with 4-NQO, compared to incubation in the absence of mutagen agent (control) screened by the macroarray technique

To investigate the combined effect of microwaves exposure and the genotoxic agent, the specific expression level of these genes was further examined by real-time RT-PCR analysis. Non exposed (sham) and exposed (PW and CW) cell culture plates (N=6 for each condition) were incubated simultaneously in three identical incubators for sham, CW and PW exposure, in the presence of 4-NQO (1.25 mM, 2 h), under gentle agitation, at 37°C followed by 1h recovery in culture medium without 4-NQO. Each experiment was reproducibly repeated 3 times. The electromagnetic field was applied alternatively in the three anechoic chambers in order to avoid cage effects.
Statistical analysis of the data was performed with the Newman-Keuls test (two factors ANOVA). No significant effect of microwave exposure was detected, either with CW or with PW (Table1).

Table 1. Real time RT-PCR quantification of the expression level of APEX, ATRX, RAD52 and MSH6 (relative units) in 4-NQO treated THP1 cells exposed to PW and CW electromagnetic field or not (sham).

Gene	**Exposure condition**				
	Sham	**CW**		**PW**	
	Expression	Expression	*p values*	Expression	*p values*
APEX	1.60 ± 0.31	1.43 ±0.30	*0.07*	1.41 ± 0.31	*0.08*
ATRX	1.02 ± 0.22	1.05 ± 0.36	*0.71*	1.05 ± 0.30	*0.91*
RAD52	1.09 ± 0.29	1.21 ± 0.29	*0.07*	1.09 ± 0.39	*0.90*
MSH6	1.32 ± 0.23	1.31 ± 0.20	*0.85*	1.22 ± 0.23	*0.40*

CONCLUSION

For this study, four genes of interest, implicated in DNA repair, were chosen according to the modification of their expression pattern after 4-NQO treatment. In our experimental conditions, no significant effect of CW and PW 2.45 GHz microwave was observed on the reactivity of the cell DNA repair system induced by the treatment with a known mutagen. Further experiments are know realized with higher SAR values and other families of gene.

ACKNOWLEDGEMENTS

We wish to thank the Instrumentation Department of the CRSSA for technical assistance, in particular to P.A. Barraud, head of department, for his open mind and kindness, to B.A. Martz and P. Parrau for cell incubator realization and to S. Pla for assembly, improvement and maintenance of the microwave irradiation system.

REFERENCES

[1] J.A. Elder, "Review of 30 studies investigating cancer in laboratory animals exposed to radiofrequency energy," *proceedings of the Biolelectromagnetics 2005 (BioEM 2005)*, p. 302-303, Dublin, Ireland, 2005.
[2] M.L. Wong and J.F. Medrano, "Real time PCR for mRNA quantitation," *BioTechniques*, vol. 39, n°1, pp. 1-9, 2005
[3] B.D. Harfe and S. Jinks-Robertson, "DNA mismatch repair and genetic instability," *Annu. Rev. Genet.*, vol. 34, pp. 359-399, 2000.
[4] A. Collin , P. Lévêque, A. Perrin and G. Testilier, "Dosimetric analysis of 1800-MHz and 2.45-GHz *in vivo* and *in vitro* exposure setup," *proceedings of the Biolelectromagnetics 2005 (BioEM 2005)*, p. 351-352, Dublin, Ireland, 2005.
[5] K.J. Livak and T.D. Schmittgen, "Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method," *Methods*, vol.25, pp.402-408, 2001.
[6] J. Vandesompele, K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe and F. Speleman, "Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes," *Genome Biol*, vol.3, research 0034, 2002.