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Hydrogen peroxide induced by modulated electromagnetic radiation protects the cells from DNA damage

Research Article

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Abstract: It is believed that non-ionizing electromagnetic radiation (EMR) and low-level hydrogen peroxide (H_2O_2) may change nonspecific resistance and modify DNA damage caused by ionizing radiation. To check this assumption, the combined effects of extremely high-frequency EMR (EHF EMR) and X-rays on induction of DNA damage in mouse whole blood leukocytes were studied. The cells were exposed to X-rays with or without preliminary treatment with EHF EMR or low-level H_2O_2. With the use of enhanced chemiluminescence, it was shown for the first time that pulse-modulated EHF EMR (42.2 GHz, incident power density of 0.1 mW/cm^2, exposure duration of 20 min, modulation frequency of 1 Hz) induced H_2O_2 at a concentration of 4.6 ± 0.3 nM L^{-1} in physiological saline. With the use of an alkaline comet assay, it was found that the exposure of cells to the pulse-modulated EHF EMR, 25 min prior to treatment with X-rays at a dose of 4 Gy reduced the level of ionizing radiation-induced DNA damage. Continuous EHF EMR was inefficient. In turn, it was shown that low-level H_2O_2 (30-500 nM L^{-1}) protected the cells against X-irradiation. Thus, the mechanisms of radiation protective effect of EHF EMR are connected with the induction of the adaptive response by nanomolar concentrations of reactive oxygen species formed by pulse-modulated EHF EMR.

Keywords: *Extremely high-frequency electromagnetic radiation • Pulse modulation • X-rays • Hydrogen peroxide • DNA damage • Comet assay • Protective effect*

1. Introduction

According to the data from international organizations, pollution of the environment by new chemical compounds formed as a result of processing input materials and a synthesis of new chemical substances is rapidly increased. Many of these chemicals possess cytotoxic, cancerogenic, and genotoxic properties either by itself or in a combination with other environmental factors. Development of telecommunication technologies, communications, radiolocation, radio navigation entails the development of new frequency bands of radio frequency electromagnetic radiation (EMR) and increase in the power of radiation sources which eventually increases the complexity of the structure of electromagnetic signals. All this raises questions about the potential adverse effects of man-made EMR of various frequency ranges including possible genotoxicity [1,2]. Considering the possibility of accidents at nuclear facilities, as well as the use of ionizing radiation in medicine, the probability of contact of humans and animals with ionizing radiation is increased. Nowadays, biological systems are simultaneously exposed to a large number of potentially genotoxic environmental factors including chemical agents, ionizing and non-ionizing EMR all of which could induce DNA damage [3,4]. Additive, synergistic, antagonistic, and potentiative effects can be observed when these factors are combined. The sequence of exposure (prior, follow, or simultaneously) and the initial functional state of the exposed biological system can have a strong dependence on the outcome.

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Protection of living organisms against the damaging action of EMR is a real and extremely complex problem of modern electromagnetic biology and radiobiology. In modern radiobiology there are many facts testifying that the radio resistance of an organism can be changed under the influence of non-radiation factors of different nature [5]. However, the question on the role of non-ionizing EMR in changing nonspecific resistance and realization of radiobiological effects are poor investigated. In this aspect the study of the mechanisms of combined effects of ionizing and non-ionizing EMR on living organisms is of great interest.

Earlier we have shown that continuous and pulse-modulated extremely high-frequency electromagnetic radiation (EHF EMR) at low intensities is able to exert positive effects in animal models of pathology. We have shown that the exposure of animals with local and systemic inflammation to EHF EMR with certain physical parameters induced pronounced anti-inflammatory effects [6,7]. The anti-inflammatory effects of EHF EMR were accompanied with significant decrease in DNA damage of peripheral blood leukocytes and neutrophils [8]. On the basis of these findings, we hypothesized that EHF EMR can induce defensive effects at the level of cellular DNA, thus protecting DNA from damaging action of different physical, chemical and biological agents. To test this hypothesis, the present study was performed.

The purpose of the study was to define the combined effects of different physical and chemical factors (agents of oxidative stress, non-ionizing and ionizing EMR) on the integrity of cellular DNA in normal blood leukocytes. Particularly, we investigate the ability of low-intensity EHF EMR at continuous wave and pulse-modulated generation mode to protect the DNA of mouse whole blood leukocytes against the damaging actions of X-rays.

2. Experimental Procedures

2.1 Chemicals

Sterile phosphate buffered saline (PBS) were prepared from 136.7 mM L^{-1} NaCl, 2.7 mM L^{-1} KCl, 8.1 mM L^{-1} Na_2HPO_4, and 1.5 mM L^{-1} KH_2PO_4 (pH=7.2). Low-melting-point (LMP) agarose was purchased from Serva Electrophoresis GmbH (Germany). Lysing solution (2.5 M L^{-1} NaCl, 100 mM L^{-1} EDTA, 1% sodium lauroylsarcosine, 10 mM L^{-1} Tris-HCl, pH=10, and 1% Triton X-100), alkaline solution (0.3 M L^{-1} NaOH, 1 mM L^{-1} EDTA; pH>13.0), and staining solution (1 μg mL^{-1} ethidium bromide in PBS) were used for the comet assay. “Count solution” containing 10 mM L^{-1} Tris-HCl buffer, pH=8.5, 50 μM L^{-1} *p*-iodophenol (Aldrich), 50 μM L^{-1} luminol (AppliChem, Germany), and horseradish peroxidase (1 nM L^{-1} for nanomolar hydrogen peroxide measurement) was used for determination of hydrogen peroxide. All chemicals were purchased from Sigma (St. Louis, MO, USA) unless stated otherwise.

2.2 Animals and blood samples

Adult male BALB/c mice (2 months of age, 22–25 g in body weight) were purchased from the Laboratory Animal Breeding Facility (Branch of Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Pushchino, Moscow Region, Russia) and used in all experiments. The mice were housed in an air-conditioned room with a controlled 12-h light–dark cycle and free access to standard chow and tap water. All manipulations with the animals were conducted in accordance with experimental protocols approved by the Local Animal Care and Use Committee (Branch of Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Pushchino, Moscow Region, Russia).

Samples of peripheral blood was collected from the tail vein of the mice into Eppendorf tubes contained PBS with 1 mM L^{-1} EDTA as anti-coagulating agent. The blood was diluted 1:7 to achieve a final concentration of leukocytes of approximately 1×10^6 cells/ml.

2.3 EMR exposure and treatment with hydrogen peroxide

A high frequency generator (G4-141; Istok, Fryazino, Russia) was the source of EHF EMR. Whole blood leukocytes embedded in agarose slides were exposed to EHF EMR in the far-field zone of a pyramidal horn antenna with an aperture of 32×32 mm^2 at a distance of 300 mm from the radiating end of the antenna. The breadth of the directional diagram of the electric field vector for the pyramidal horn antenna was $2\theta^E_{0.1}$ ~ 25°. Accordingly, the major lobe width (0.1 level) was about 130 mm at a distance of 300 mm from the antenna [9]. To eliminate the interference in the plane of an exposed object, an effective multilayer absorbent was placed between the slides and the table; therefore, the conditions of exposure were close to free-field conditions. Exposure conditions and parameters of EHF EMR whose high efficacy has been shown previously [7] were used: carrier frequency of 42.2 GHz, incident power density of 0.1 mW/cm^2, continuous generation mode or pulse modulation by a meander signal, a positively defined rectangular wave signal with 50% duty factor, at fixed frequency of 1 Hz. Dosimetric tests were conducted as described earlier [9,10]; the surface specific absorption rate was about 1.5 W/kg at an incident power density of 0.1 mW/cm^2. Sham-exposure procedures were conducted by placing the slides into the

exposure zone when the generator was turned on but the output power was maximally attenuated (to < 1 μW). In all these experiments, the duration of the exposure and sham exposure was 20 min at room temperature, 25 min prior to X-irradiation. The background induction of the geomagnetic field was 45±3 μT.

Whole blood leukocytes embedded in agarose slides were exposed to X-rays at a dose of 4 Gy (dose rate of 1 Gy min^{-1}) from a RUT-250-15-1 therapeutic X-ray device (Mosrentgen, Moscow, Russia) at room temperature. In a separate series of experiments, whole blood leukocytes embedded in agarose slides were treated with hydrogen peroxide by placing the slides into solution of hydrogen peroxide at concentrations of 10, 30, 50, 100, 300, 500, 1000, 2000, and 5000 nM L^{-1} for 10 min at 37°C 15 min prior to X-irradiation. The concentrations were selected considering that concentrations less than 1000 nM L^{-1} did not cause DNA damage in our experiments and assuming that the concentrations of hydrogen peroxide that could be produced by EHF EMR exposure directly in the cells may be higher than the concentrations penetrating into the cells of exogenous hydrogen peroxide due to an antioxidant activity of biological molecules.

2.4 Alkaline comet assay

To assess DNA damage in mouse blood leukocytes, we used the alkaline comet assay (single-cell gel electrophoresis) [11-13] with some modifications [8]. An aliquot of the diluted blood was mixed at 37°C with an equal volume of 1% LMP agarose prepared in PBS with 1 mM L^{-1} EDTA. To prepare three-layer agarose slides, 5-10 μl of the mixture were spread (under a cover slip) on each glass slide precoated with 0.5% LMP agarose. After solidification of agarose with the cells for 5 min at 4°C, an additional layer of 5-10 μl of 0.5% LMP agarose was added and solidified for 5 min at 4°C. Ready agarose slides were subjected to the comet assay procedures [8] after different treatments. Specified number of slides were prepared from each blood sample according to the number of treatments. Routinely, a total of about 50 cells per slide were registered and DNA damage was assessed on percentage of DNA in a comet tail (tail DNA). Mean values and standard errors of the mean (SEM) for each treatment were calculated from independent experiments (animals) (n = 9).

2.5 Determination of hydrogen peroxide

The production of hydrogen peroxide in PBS exposed to EHF EMR was measured using a sensitive assay based on enhanced chemiluminescence in a peroxidase–luminol–*p*-iodophenol system [14]. The chemiluminescence was quantified with a Beta-1 liquid scintillation counter (Manufacturing Association Medapparatura, Ukraine). The counter was operated in the single photon counting mode with one photomultiplier and with coincidence scheme disengaged. Samples of PBS (7 ml each) were exposed to EHF EMR (under the same conditions as agarose slides) in cylindrical polyethylene containers with a diameter of 50 mm placed in the far-field zone of a pyramidal horn antenna. To measure chemiluminescence, an aliquot of 3 ml of exposed PBS was taken and 3 μl of "count solution" was added in 20-ml liquid scintillation counter vials (Beckman). Five or more samples of PBS were measured in each experiment.

2.6 Statistical analysis

All experiments were conducted utilizing the “blind” experimental protocol, where the investigator making the measurements did not know which treatments were made. The data were systematized according to the type of treatment. All data are given as the mean ± SEM. The normality of data was analyzed using the Kolmogorov-Smirmov test. All the data matched the normal distribution. Comparisons between different treatments and the corresponding control were made using one-way analysis of variance (ANOVA) followed by the Dunnett’s multiple comparison test ($p < 0.01$) or Student’s t-test ($p < 0.05$) for pair-wise comparison of different groups of data.

3. Results

The aim of the study was to assess the ability of low-intensity EHF EMR at continuous wave and pulse-modulated generation mode under preliminary exposure to protect DNA of mouse whole blood leukocytes against damaging action of X-rays. We also looked at the role of reactive oxygen species (ROS), particularly hydrogen peroxide as long-lived member of ROS, in possible mechanisms of a protective effect of EHF EMR.

First of all, using an alkaline comet assay, we have studied DNA damage in mouse whole blood leukocytes exposed to different doses of X-rays. We found that the percentage of tail DNA (TDNA) depends linearly on the X-ray dose in a range of 0-10 Gy and well approximated by an equation:

TDNA (%) = 2.51 × D – 0.05, where D is the X-ray dose (in Gy).

The dose of 4 Gy was chosen for further experiments as a maximal non-lethal dose *in vivo* [15] and in our experimental conditions it induced appreciable but not dramatic level of DNA damage in mouse leukocytes *in vitro* which was about 9.9±0.4%. Then we have tested

the efficacy of two exposure modes of low-intensity EHF EMR (continuous waves and pulse-modulated with a frequency of 1 Hz) to induce radiation protective effects against X-irradiation at a dose of 4 Gy. The exposure of cells to EHF EMR in any mode did not lead to noticeable changes in DNA damage when compared to the sham-exposure control (Figure 1). Preliminary sham-exposure or exposure of cells to continuous EHF EMR before treatment with X-rays did not induce radiation protective effects (Figure 1). The preliminary exposure of cells to pulse-modulated EHF EMR with modulation frequency of 1 Hz reduced the X-irradiation induced DNA damage on an average to 7.8 ± 0.4% (p <0.01), the dose reduction factor is about 1.27. Thus, at the fixed carrier frequency of 42.2 GHz and incident power density of 0.1 mW/cm^2, the radiation protective effect was observed only under exposure to pulse-modulated EHF EMR (modulation frequency of 1 Hz), and continuous EHF EMR was inefficient.

We hypothesized that the mechanisms of radiation protective effect of pulse-modulated EHF EMR is related to the induction of adaptive response by low concentrations of ROS. Previously, it was found that the non-ionizing EMR could form ROS in aqueous systems [16-18], which, in turn, may induce an adaptive response. We have shown that the exposure of phosphate buffered saline to pulse-modulated EHF EMR (42.2 GHz, 0.1 mW/cm^2, 20 min, modulation frequency of 1 Hz) leads to the production of hydrogen peroxide in concentration of 4.6 ± 0.3 nM L^{-1} while continuous EHF EMR did not produce hydrogen peroxide.

In a separate series of experiments the possibility of induction of adaptive response in mouse leukocytes pretreated with exogenous hydrogen peroxide at nanomolar concentrations (10 – 5000 nM L^{-1}) to the X-irradiation at a dose of 4 Gy was tested. These experiments showed that the incubation of whole blood leukocytes for 10 min at 37°C in the presence of hydrogen peroxide at concentrations of up to 1 μM L^{-1} did not induce the significant increase in DNA damage. Hydrogen peroxide at a concentration of 1 μM L^{-1} resulted in approximately 3-fold increase in DNA damage from 0.12 ± 0.06% in the control to 0.37 ± 0.08% (p <0.02). Incubation of whole blood leukocytes in the presence of hydrogen peroxide in concentrations of 2 and 5 μM L^{-1} induced gradual increase in the DNA damage, which was about 0.42 ± 0.05% (p <0.003) and 0.76 ± 0.18% (p <0.001), respectively. When looking at the combined action of exogenous hydrogen peroxide and X-rays, we found that the pretreatment of cells with hydrogen peroxide at concentrations of 30 – 500 nM L^{-1} (for 10 min at 37°C) leads to significant reduction in DNA damage after subsequent X-irradiation at a dose of 4 Gy (Figure 2). The effect has a bell-shaped dependence on the priming concentration of hydrogen peroxide, with a maximal effect seen at concentrations of 100-300 nM L^{-1}.

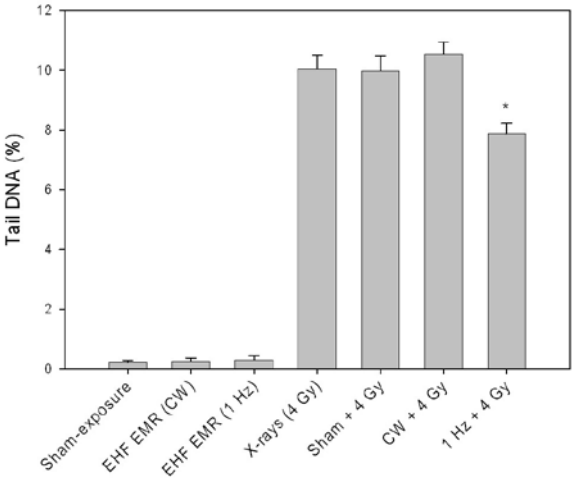

Figure 1. DNA damage in mouse whole blood leukocytes under different exposure conditions: sham-exposure, exposure to continuous EHF EMR, exposure to pulse-modulated EHF EMR with modulation frequency of 1 Hz, X-irradiation at a dose of 4 Gy, combined sham-exposure and X-irradiation, combined exposure to continuous EHF EMR and X-irradiation, and combined exposure to pulse-modulated EHF EMR and X-irradiation. * – statistically significant difference compared to other experimental groups (p < 0.01 by the Dunnett's multiple comparison test); n = 9.

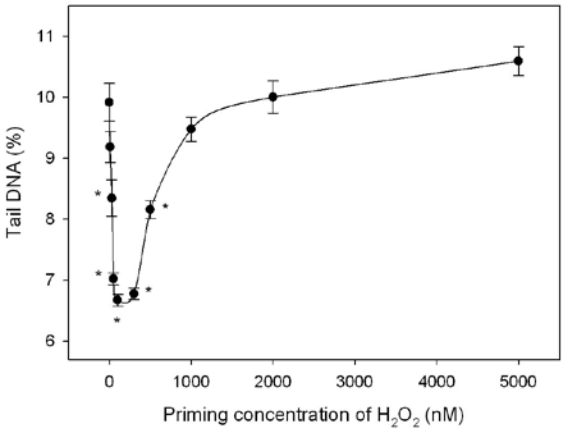

Figure 2. DNA damage in mouse whole blood leukocytes when pretreated with hydrogen peroxide at various concentrations and subsequently exposed to an X-ray at a dose of 4 Gy. * p <0.005 compared to the control (0 nM) by the Student's t-test; n = 9 for each point.

4. Discussion

The adaptive response is one of the mechanisms of the radiation protective effect and is a universal response of cells to irradiation at small doses; the effect of which is manifested by an increase in resistance to the damaging effects of radiation at larger doses or other non-radiation agents [19,20]. Adaptive response in biomedicine underlies the protective effect of various agents with mutagenic and genotoxic properties [21,22]. According to many researchers, the mechanisms of adaptive response are connected to cell repair systems [5,23]. Adaptive responses can be triggered and switched by various inducers of stress, including high temperature and other physical factors, biologically active substances, as well as ROS. Compared with other members of ROS, hydrogen peroxide is less toxic, long-lived, and is able to diffuse through biological membranes and reach different targets in their natural environment [22]. Therefore, hydrogen peroxide is often applied as the representative ROS in modeling oxidative stress.

The data available in the literature indicates that low concentrations of hydrogen peroxide can stimulate *de novo* synthesis and expression of the antiapoptotic protein Bcl-2 [22]. Bcl-2 can counteract the pro-apoptotic effect of Bax and then enhance cell survival. An increased Bcl-2/Bax ratio in cells could suppress the cleavage of poly(ADPribose) polymerase 1 (PARP-1), which is a nuclear enzyme that responds to DNA damage, facilitates DNA repair, and promotes cell survival [24]. Based on these findings we suggest that in our experiments, cellular responses to nanomolar concentrations of hydrogen peroxide might be similar, but their role in protection of the cells against ionizing radiation should be further investigated. Low level of hydrogen peroxide similar to low-intensity EHF EMR, which is not genotoxic *per se*, may induce a small amount of DNA damage priming DNA repair and preparing it to receive a challenge dose.

Unfortunately, the detailed mechanisms of induction of adaptive response are not currently well understood. It was found that micromolar concentrations of hydrogen peroxide can induce *de novo* synthesis of a large numbers of proteins that are involved in energy metabolism, signaling, DNA translation, transcription and repair, regulation of redox potential, stress responses, apoptosis, protein folding, *etc.* [22,25]. It has been shown that hydrogen peroxide at micromolar concentrations blocks the activation of stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) and associated signaling pathways [26]. In addition, previous studies have shown that micromolar concentrations of hydrogen peroxide are capable of activating some antioxidant enzymes, including catalase and glucose-6-phosphate dehydrogenase [27]. These and other experimental findings indicate that the adaptive response is a complex reaction involving a wide range of cellular functions.

Thus, we have shown that low-intensity EHF EMR with certain physical parameters can provide the radiation protective effect, the mechanisms of which may be connected with the induction of the adaptive response. The adaptive response can be induced by low concentrations of ROS produced under the exposure to pulse-modulated EHF EMR. It was found that exogenous hydrogen peroxide in nanomolar concentrations (30 – 500 nM L^{-1}) are capable of inducing the adaptive response in mouse whole blood leukocytes thus, protecting the cells from the subsequent exposure to high dose of X-rays. The revealed mechanism can underlie radiation protective effects of non-radiation factors, including low-intensity EHF EMR. Clarification of the mechanisms of modifying effects of non-ionizing EMR against the background of increased background radiation and chemical treatments is extremely important from the point of view of hygienic standardization of EMR and their application in biomedicine. The obtained new knowledge will contribute to the development of strategies and means for protection against EMR and damaging concentrations of chemical agents.

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