Decrease of reactive oxygen species (ROS) production by neutrophils after incubation in hypomagnetic conditions

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Abstract. Incubation of the suspension of neutrophils in hypomagnetic field generated by a system of magnetic shields (residual constant magnetic field not exceeding 20 nT) leads to significant decrease in reactive oxygen species (ROS) production compared to control (geomagnetic field 44 μT), which was recorded by lucigenin-dependent chemiluminescence and fluorescent spectroscopy with 2,7-dichlorodihydrofluorescein diacetate (H₂DCF-DA). During increase of constant magnetic field (CMF) in 0.02–44 μT range, polyextreme character of the response of the neutrophils to this action was observed: the minima of ROS production were at 0.02 μT and 7.0 μT, alternating with 2.5 μT and 30 μT values, at which the used test system does not react to the exposure to CMF.

1. Introduction

Many authors regard the possibility of effect of weak magnetic fields on reactive oxygen species (ROS) production and generation of other free radicals as a promising approach to the analysis of mechanisms of their biological action [1–3].

There is a number of reports in the scientific literature about the effect of weak low-frequency MFs on the kinetics of ROS formation in the suspension of neutrophils activated by chemical stimulators of respiratory burst [4–8]. It was also reported on the effect of hypomagnetic conditions (HMC) on ROS production in different cells [9–13].

We have earlier shown that exposure of murine peritoneal neutrophils to magnetic shielding in HMC causes the decrease of intracellular ROS production recorded by change of fluorescence intensity of the products of 2,7-dichlorodihydrofluorescein and dihydrorhodamine 123 [14]. In these experiments, we found that the effect of the hypomagnetic field is apparent on neutrophils without their additional stimulation by the chemical activators of respiratory burst, and, consequently, the mechanism of such action can be not related to the impairment of the response of neutrophils to these stimuli. Thus, in this work we carried out a complex study on non-activated neutrophils to determine possible molecular mechanisms and targets of the “zero” magnetic field action.

We must note that the aforementioned results were obtained using the method of fluorescence spectroscopy with intensely reacting fluorescent probes that are not selective to certain ROS types (2,7-dichlorodihydrofluorescein diacetate and dihydrorhodamine 123) [15–18]. In this work, we also used another method, activated chemiluminescence with selective probe for superoxide anion, lucigenin [19,20], to estimate the radical producing ability of the neutrophils after exposure to HMC.
Attention was paid to determination of residual constant magnetic field (CMF) values at which the effects of HMC can be reproduced.

2. Materials and methods

The work was carried out on murine peritoneal neutrophils. Male laboratory mice CD-1 (bodyweight 22-25 g) obtained from the nursery of the Shemyakin—Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences were used for isolation of the cells. The mice were administered opsonized Zymozan A suspension (150 μl i.p., 5 mg/ml) (Zymozan A from \textit{Saccharomyces cerevisiae}, Sigma, USA). After that, the animals were brought to an end by cervical dislocation 12 hours after administration, four milliliters of calcium-free Hank’s solution were injected into their abdominal cavity for washing [5]. The exudate was collected by pipetting, and the cell pellet was diluted in 4 ml of calcium-free Hank’s solution and incubated for 1 hour at 4°C [5]. The isolated cells were counted in Goryaev chamber. Cell viability was determined using the vital dye, trypan blue (Sigma, USA). Content of living cells comprised at least 98%. The experimental samples were obtained by dilution of the suspension with Hank’s medium (138 mM NaCl, 6 mM KCl, 1 mM MgSO\(_4\), 1 mM Na\(_2\)HPO\(_4\), 5 mM NaHCO\(_3\), 5.5 mM glucose, 1 mM CaCl\(_2\), 10 mM HEPES, pH 7.4; Sigma, USA) to 1∙10\(^6\) cells/ml concentration.

The procedures followed were approved by the ethics committee for guidance for care and use of laboratory animals № 57.30.12.2011 of the Institution and in accordance with the Guidelines for Ethical Conduct in the Care and Use of Animals.

The neutrophils were incubated at 37.0\(\pm\)0.2°C at 10\(^6\) cells/ml concentration in 0.25 ml aliquots in round-bottom polystyrene cuvettes (d = 1.2 cm, l = 5.5 cm) (Sarstend, Germany), in which further fluorescence measurements were taken. Typical incubation time was 40 min. The set temperature was maintained by a circulation thermostat UH 4 (MLW, Germany).

The control samples were placed in local geomagnetic field (GMF) with a constant component around 44 μT and 50 Hz technogenic magnetic background around 15-50 nT at the same time and temperature as the experimental ones. The experimental specimens were placed into an appliance for maintaining hypomagnetic conditions.

An appliance for creation of HMC used in the work was capable of decreasing the magnetic field by four orders of magnitude (the residual field did not exceed 20 nT) and it significantly mitigated alternating technogenic noise (to several nT) [8]. The appliance was composed of three coaxial cylindric magnetic shields made of 1 mm thick permalloy. The residual fields inside the appliance were measured directly with ferroprobe magnetometer, Mag – 03 MS 100 (Bartington, UK). The experimental weak uniform constant magnetic field was generated by a special inductor (solenoid) installed inside the appliance. The solenoid could be attached to a direct current source to form weak magnetic field of various intensities (2.5; 7; 30; 44 μT) used in a series of experiments. Solenoid dimensions were: diameter, 18 cm; length, 38 cm (720 coils of copper wire, diameter 1 mm; the resistance of solenoid was 7.5 Ohm). The size of experimental area inside the shields allowed simultaneous placement of a number of samples sufficient for the experiment (at least 6) into the uniform CMF zone. The experiments were performed in triplicates.

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After the incubation of the neutrophil suspension in control and experimental groups, a solution of lucigenin (Enzo Life Sciences, USA) was added at a final concentration of 0.35 mM. The measurements of luminescence were performed on a 12-channel chemiluminometer Lum-1200 (DISoft LLC, Russia). There was a linear correlation between values of luminescence intensity and number of photons per time unit: 1 Volt of recorded voltage corresponded to ≈ 1000 photons/s. Chemiluminescence records were analyzed in PowerGraph program. Some results were expressed in relative units (percent of control chemiluminescence amplitude, which was taken for 100%). After 40 min incubation, a part of the samples of neutrophil suspension was supplied with fluorescent probe for intracellular ROS, 2,7-dichlorodihydrofluorescein diacetate (Sigma, USA) to a final concentration of 0.01 mg/ml. The samples were incubated for extra 25 min at 37°C in the dark to minimize the
photooxidation of the dye. Then, the cells were transferred to Eppendorf tubes, washed by centrifugation at 600 g for 5 min in Hank’s solution. The pellet was then resuspended in 1 ml of the medium and fluorescence spectra of the specimens were recorded on Thermo Scientific Lumina Fluorescence Spectrometer (Thermo Fisher Scientific, USA) at 488 nm excitation wavelength. A part of the results was represented as percentage of fluorescence intensity at 528 nm in the control group, which was taken for 100%.

The data were evaluated using Student’s test. P-values < 0.05 were considered significant. All values were expressed as means ± SD.

3. Results and discussion
Pre-incubation of the neutrophil suspension in “zero” magnetic field (<0.02 μT) led to significant decrease of the intensity of lucigenin-dependent chemiluminescence (by around 30%) (figure 1, figure 2). When the constant field was increased to 2.5 μT, this effect vanished, but at CMF value = 7 μT it appeared again; at 30 and 44 μT, the effect was absent (the value corresponded to CMF value in control) (figure 2). Such polyextreme character of the dependence of response to weak CMF was also noticed in the experiment on neutrophils during ROS production registration by fluorescence spectroscopy (figure 3, figure 4). It is important that this result was obtained by two different methods, lucigenin-dependent chemiluminescence and fluorescence spectroscopy, which does apparently increase its reliability level.

The discovered dependence of ROS production in neutrophils on the value of constant magnetic field in the studied induction range (0.02 - 44 μT) evidencing on high sensitivity of such processes to changes of magnetic conditions appears to be of high information value. The revealed anisotropy of the response of biological system on such an action could give certain data for analyzing the physical mechanisms of non-specific magnetoreception. In the current moment, a model of non-specific (not related to certain receptors) magnetoreception becomes more popular [21]. It is based on the analysis of precession of magnetic moments and molecular rotations in weak MF, and our data could be useful.

Figure 1. The kinetics of the chemiluminescence response of the neutrophil suspension to lucigenin after exposure to “zero” magnetic field (CMF < 0.02 μT).

Figure 2. The influence of a constant MF on the intensity of the lucigenin-dependent chemiluminescence in the neutrophil suspension. Statistically significant differences from the control values are pointed by an asterisk (P < 0.05).
for its realistic validation. Moreover, these data could be useful for estimation of quantum mechanical model based on application of spin chemistry for the cases of weak MFs [22], which gives a prediction on reaction product (peroxyradical) yield depending on MF parameters, allows to estimate the contribution of weak MF to this process, and shows the polyextreme character of the response of biological system depending on the magnitude of MF.

![Fluorescence intensity vs. wavelength](image1.png)

**Figure 3.** The fluorescence spectra of dichlorofluorescein in neutrophil suspension after exposure to “zero” magnetic field (CMF < 0.02 μT).

From the practical point of view, the result showing the absence of biological action of weak CMF at 2.5 μT, while the neutrophil suspension reacts both at lower (0.02 μT) and higher (7 μT) magnitudes, is important. This fact is also interesting because earlier the similar result (absence of action) was shown at close CMF value, 3 μT, on another experimental model, division of planaria *Dugesia tigrina* [23].

There are several cellular systems generating free radicals as main products or byproducts. First of all, such systems include NADPH oxidases, membrane-bound enzymes producing superoxide anion radical (SAR) via one-electron reduction reaction [24]. Beside that, an important system of SAR production are mitochondria. The leakage of SAR was shown to occur in 11 sites of their inner membrane, mainly in complexes I, II and III, SAR being produced both into matrix and into intermembrane space [25]. Lucigenin is considered as a selective probe for SAR [19], this is why it is actively used for studying ROS production by both NADPH oxidase and mitochondria [20]. We have previously shown [26] that the addition of a NADPH-oxidase inhibitor diphenyliodonium to the incubation medium leads to the decreased intensity of chemiluminescence in both experimental (hypomagnetic conditions) and control (geomagnetic field) samples. The differences between the groups caused by action of “zero” magnetic field were observed in a wide range of concentrations of this inhibitor (2.5-100 μM) to the similar extent. Contrary to that, addition of an agent uncoupling oxidation and phosphorylation in mitochondria, 2,4-dinitrophenol, from 5 μM and up to 200 μM, almost completely leveled the differences between control and experimental group, that were observed in absence or at lower concentrations of this inhibitor. These data show the prospect of studying neutrophil mitochondria as potential targets reacting on changes of CMF values.

On the whole, the obtained data show high sensitivity of biological processes to HMC and variations of weak static magnetic fields.

![Fluorescence intensity vs. magnetic field](image2.png)

**Figure 4.** The influence of a constant MF on the intensity of dichlorofluorescein fluorescence in the neutrophil suspension. Statistically significant differences from the control values are pointed by an asterisk ($P < 0.05$).
4. Conclusion

Two different methods, fluorescence spectroscopy determining intracellular ROS production, and chemiluminescence analysis with lucigenin, a SAR-specific chemiluminescence activator, have shown a decrease in ROS production by neutrophils under hypomagnetic conditions. This effect of hypomagnetic field depends on the magnitude of the residual constant magnetic field in the same way for both research methods used. It must be noted that both methods for ROS detection used in the work revealed one value of weak constant magnetic field, 2.5 µT, under which the studied system (neutrophil suspension) makes no significant response to the field compared to ROS production under natural geomagnetic field (44 µT). Meanwhile, the changes of weak constant field value in the area close to 2.5 µT (switching to 0.02 µT or 7 µT) led to decrease in ROS production also detectable by both methods. Since the value of the acting magnetic field can be related to the value of magnetic moment of the field sensor and its lifetime, these results could be useful for discovering the sensors of weak magnetic field in the studied biological system.

5. References

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