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CELL BIOPHYSICS

On the Molecular Mechanisms of the Effect of a Zero Magnetic Field on the Production of Reactive Oxygen Species in Inactivated Neutrophils[1]

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Abstract—It is shown that the lower intensity of 2,7-dichlorodihydrofluorescein oxidation processes in inactivated neutrophils exposed to hypomagnetic field (the residual static magnetic field 20 nT) is not related to calcium-mediated mechanisms as shown by the absence of the effect of cell-permeant Ca^{2+} chelators, such as 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis(acetoxymethyl ester), on the intensity of the process. This decrease is hardly likely to be caused by effects of a hypomagnetic field on phosphorylation of NADPH-oxidase components, because addition of the protein kinase C inhibitor Ro 316233 decreases the fluorescence intensity of intracellular dichlorodihydrofluorescein little, if at all. Addition of phospholipase C inhibitor U73122 causes a negligible decrease in ROS production in the control and experiment, almost equally. Different concentrations of apocynin increase ROS production in nonactivated neutrophils and this effect is approximately two times lower under hypomagnetic conditions. The decrease in ROS production is more pronounced in cells treated with a hypomagnetic field with the presence of rotenone, indicating that the mitochondrial electron-transport chain is involved in the mechanism of the effect of hypomagnetism.

Keywords: hypomagnetic field, neutrophils, reactive oxygen species, fluorescence, calcium ions, protein kinase C, phospholipase C, mitochondria

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There were several reports on lower production of reactive oxygen species (ROS) under hypomagnetic conditions in various cell types and at various exposure regimes [1–4]. Previously, we showed that the magnetic screening of murine peritoneal neutrophils (residual constant magnetic field below 20 nT) for 1.5 h caused a decrease in basal intracellular ROS production, assessed by changes in the fluorescence of oxidation products of 2,7-dichlorodihydrofluorescein and dihydrorhodamine 123 [5]. This effect of a hypomagnetic ("zero") field remained with the presence of small concentrations of the respiratory burst activator, formylated peptide N-formyl-Met-Leu-Phe or phorbol 12-myristate 13-acetate [5]. With regard to the fact that the hypomagnetism effect manifested itself in neutrophils without additional stimulation, thus being caused by other factors than the impaired response of neutrophils to respiratory burst activators, we undertook a series of experiments with nonactivated neutrophils to identify putative molecular mechanisms that underlie the effect of zero field. In contrast, experiments with combined magnetic fields with certain parameters [6, 7] reveal a stimulatory effect of these magnetic fields on ROS production in neutrophil suspension [8]. In this regard, comparison of the key aspects of the molecular mechanisms mediating the action of combined magnetic fields [9–12] and zero magnetic field is of special interest.

At this phase of the study we employed fluorescence spectrometry with the well-studied cell-permeant ROS probe, 2,7-dichlorodihydrofluorescein diacetate (H2DCF-DA) [13–16] for studying the effects of hypomagnetism. The probe penetrates into the cell, where it is deacetylated to H2DCF by intracellular esterases. H2DCF fluoresces weakly, but its reactions with oxidizers yield intensely fluorescing dichlorofluorescein.

The chief ROS producers in nonactivated neutrophils are the NOX2-containing phagocyte oxidase complex and occasional mitochondria [17–19]. The NADPH oxidase assembly and activity directly depend on the activities of phospholipase C and pro-

Abbreviations: ROS, reactive oxygen species, H2DCFDA, 2,7-dichlorodihydrofluorescein diacetate; BAPTA AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis(acetoxymethyl ester).

tein kinase C. They are often conjugated with calcium-mediated regulatory mechanisms [20]. Therefore, we used appropriate chemical inhibitors for analysis of the molecular mechanism of the effect of hypomagnetism on ROS production in nonactivated neutrophils.

MATERIALS AND METHODS

Preparation of the neutrophil suspension. Experiments were carried out with peritoneal neutrophils isolated from 24–26 g male CD-1 laboratory mice. The mice were received from the vivarium of the Branch of the Shemyakin–Ovchinnikov Institute of Bioorganic Chemistry, Pushchino, Moscow oblast, Russia. Opsonized zymosan (Zymosan A from *Saccharomyces cerevisiae*, Sigma, United States; 5 mg/mL, 150 μL) was injected intraperitoneally. Twelve hours later, the animals were euthanized by ulnar dislocation and the abdominal cavity was flushed with 3 mL of cooled calcium-free Hanks' solution. The exudate was collected with a pipette and centrifuged at 600 g for 10 min. The supernatant was poured off and the sediment was dissolved in 2 mL of calcium-free Hanks' solution and incubated at 4°C for 1 h. Cells were counted in a hemocytometer. Cell viability was tested with the vital stain trypan blue. The percentage of living cells was no less than 98%. Samples for experiments were prepared by diluting neutrophil suspensions with standard Hanks' solution (138 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1 mM Na_2HPO_4, 5 mM $NaHCO_3$, 5.5 mM glucose, 1 mM $CaCl_2$, 10 mM HEPES pH 7.4 Sigma, United States) to the concentration 10^6 cells/mL.

Exposure of neutrophil suspension to hypomagnetic field. Neutrophils were incubated in Eppendorf-compatible polypropylene tubes at the concentration of 10^6/mL in the volume 250 μL in darkness. The temperature was controlled with a circulation bath. Typically, the incubation lasted for 1.5 h. Control samples were kept in the local geomagnetic field with the steady component of approximately 42 μT and magnetic noise 15–50 nT at 50 Hz.

The device that provided the hypomagnetic conditions consisted of three 1-mm thick coaxial cylindrical permalloy magnetic field screens. The residual constant magnetic field measured with a Mag-03 MS 100 ferroprobe (Bartington, United Kingdom) did not exceed 20 nT. Control (geomagnetic field) and experimental (hypomagnetic field) samples, ten in each group, were incubated simultaneously. The experiments were conducted in no less than three replications.

Prior to incubation, some samples were individually supplemented with various chemicals: cell-permeant calcium-chelating agent 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA AM; Sigma, United States), final concentrations 2 or 4 μM; inhibitor of the mitochondrial electron transport chain rotenone (Sigma, United States), 1 μM; inhibitor of NADPH oxidase apocynin, 20 or 500 μM; phospholipase C inhibitor U73122 (Sigma, United States), 2 μM; and protein kinase C inhibitor bisindolylmaleimide IV (Ro 31-6233, Sigma, United States), 1 μM.

Fluorescence assay of intracellular ROS. After 1.5-h hypomagnetic treatment of neutrophil suspension, the ROS probe H2DCF-DA (Sigma, United States) was added to the final concentration of 0.01 mg/mL. The samples were further incubated in darkness, to minimize dye photooxidation, at 37°C for 30 min. The cells were then washed with Hanks' solution by centrifuging them at 600 g and room temperature for 10 min. One milliliter of the medium was added to the sediment and resuspended. Fluorescence spectra were recorded with a Thermo Scientific Lumina Fluorescence Spectrometer (Thermo Fisher Scientific, United States) with excitation at 488 nm.

Statistical evaluation of the results was performed using the Student's *t* test. Some results are presented at the maximum fluorescence intensity at 528 nm with reference to the basal control (TN: neutrophils without additives not exposed to zero field), taken to be 100%.

RESULTS AND DISCUSSION

Hypomagnetic treatment of peritoneal neutrophils (approximately 2000-fold geomagnetic field weakening) significantly (by 25%) decreased the fluorescence of intracellular dichlorofluorescein (Figs. 1 and 2). We note that the spectrum shape and the wavelength of the maximum probe fluorescence in the zero magnetic field were the same as in the control. They also remained the same with the presence of chemicals used in subsequent experiments.

Addition of 2 or 4 μM BAPTA AM as the cell-permeant calcium chelating agent to the neutrophil incubation medium exerted practically no effect on the production of intracellular ROS by neutrophils in experimental (hypomagnetic field) or control (local magnetic field) samples (Fig. 2). This observation indicates the independence of the effect of hypomagnetism on the intracellular calcium level or on calcium-dependent mechanisms in general. It differentiates this effect from that of combined magnetic fields on the same object, neutrophils, where it is entirely inhibited even by low BAPTA AM concentrations [9].

Addition of 1 μM rotenone, an inhibitor of mitochondrial electron transport chain, reduced intracellular ROS production by 20% in the control and much more, by 35%, in the experiment (Fig. 3). This observation indicates that mitochondria are involved in the effect of the hypomagnetic field and confirms their role as one of the main ROS producers in nonactivated

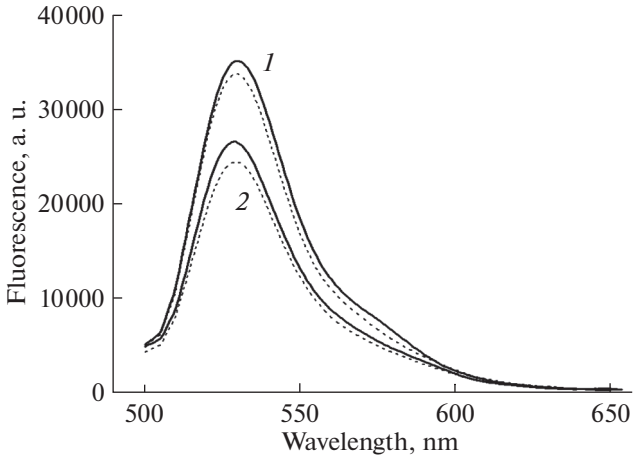

Fig. 1. Dichlorofluorescein fluorescence spectra in neutrophil suspensions: control (curve *1*) and exposed to hypomagnetic field (curve *2*). Dashed curves indicate standard deviations.

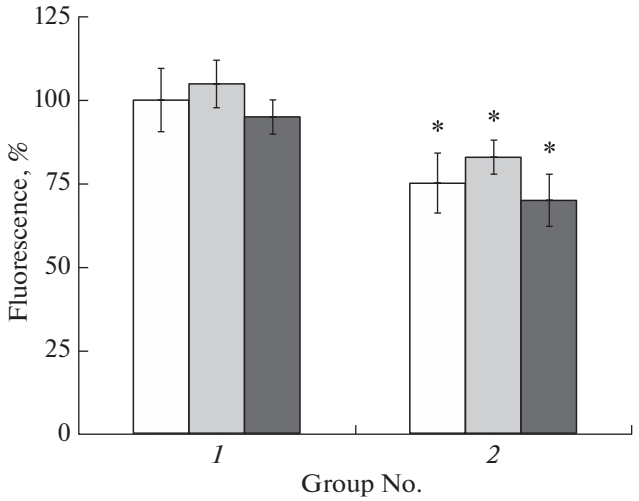

Fig. 2. The effect of a hypomagnetic field on dichlorofluorescein fluorescence in neutrophil suspensions with and without the presence of the cell-permeant calcium chelating agent BAPTA AM. Y-axis: maximum fluorescence intensity as percentage of the basal control (mean values and standard deviations, $n = 10$). X-axis: *1*, control group; *2*, experiment. Open bars: no additives; gray bars: 2 μM BAPTA AM; dark bars: 4 μM BAPTA AM. * The differences between groups are significant at $p < 0.05$.

neutrophils [17, 18]. It should be mentioned in this regard that the effect of combined magnetic fields does not depend on the addition of this inhibitor to the neutrophil culture medium [12].

Addition of the phospholipase C inhibitor U73122 reduced ROS production in the experiment and control approximately equally, by 20% (Fig. 4). Addition of the protein kinase C inhibitor Ro 31-6233 exerted practically no effect on intracellular dichlorofluorescein fluorescence (Fig. 5). These data are consistent with the aforementioned independence of the zero-field effect on calcium-mediated regulatory mechanisms. They do not confirm the involvement of phosphorylation of NADPH oxidase components in the mechanisms of the effect.

Addition of various concentrations of the specific NADPH oxidase inhibitor apocynin caused a paradoxical increase in ROS production in nonactivated neutrophils. The increase was in direct proportion to inhibitor concentration and was approximately twice as large as that in the control samples (Fig. 6). This phenomenon can be explained on the basis of the fact that the NADPH oxidase-inhibiting activity of apocynin requires its dimerization, which occurs in the presence of peroxidases and hydrogen peroxide [21, 22], e.g., in activated neutrophils. In nonactivated cells, apocynin can enhance ROS production owing to its prior oxidation, generating transient free radicals [23]. Concerning our experiments, it is pertinent to mention that the rate of this process under experimental conditions (after hypomagnetic treatment) decreases significantly.

To sum up, our results point to a decrease in the rates of oxidation of the H2DCF fluorescence probe in neutrophils under hypomagnetic treatment, involvement of mitochondria and their electron-transport chain in the zero-field effect, putative slowdown of the oxidation of some other compounds (e.g., apocynin), and independence of the zero-field effect on calcium-dependent regulatory mechanisms. Thus,

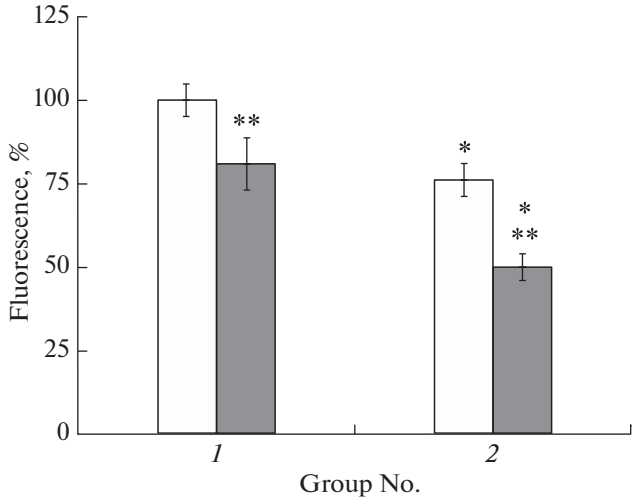

Fig. 3. The effect of hypomagnetic field on dichlorofluorescein fluorescence in neutrophil suspensions with and without the presence of rotenone. Y-axis: maximum fluorescence intensity as the percentage of the basal control (mean values and standard deviations, $n = 10$). X-axis: *1*, control group; *2*, experiment. Open bars: no additives; gray bars: 1 μM rotenone. *Differences between groups significant at $p < 0.05$. **Differences within a group significant at $p < 0.05$.

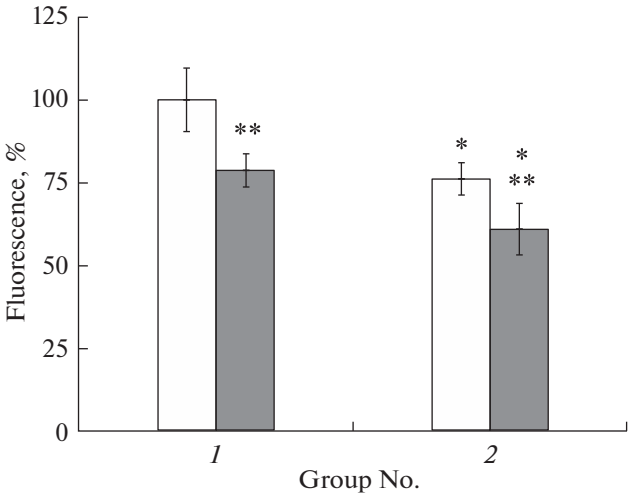

Fig. 4. The effect of a hypomagnetic field on dichlorofluorescein fluorescence in neutrophil suspensions with and without the presence of phospholipase C inhibitor U73122. Y-axis: the maximum fluorescence intensity as percentage of the basal control (mean values and standard deviations, $n = 10$). X-axis: *1*, control group; *2*, experiment. Open bars: no additives; gray bars: 2 μM U73122. * Differences between groups significant at $p < 0.05$. ** Differences within a group significant at $p < 0.05$.

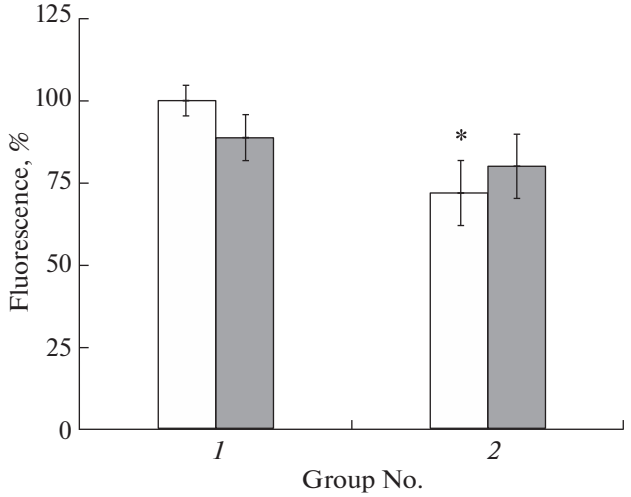

Fig. 5. The effect of hypomagnetic field on dichlorofluorescein fluorescence in neutrophil suspensions with and without the presence of protein kinase C inhibitor Ro 31-6233. Y-axis: the maximum fluorescence intensity as percentage of the basal control (mean values and standard deviations, $n = 10$). X-axis: *1*, control group; *2*, experiment. Open bars: no additives; gray bars: 1 μM Ro 31-6233. * The difference between groups is significant at $p < 0.05$.

a metabolic basis may be conjectured for this effect. In all these features, the zero-field effect differs dramatically from the effects of combined magnetic fields with certain parameters [6, 7], which show an association

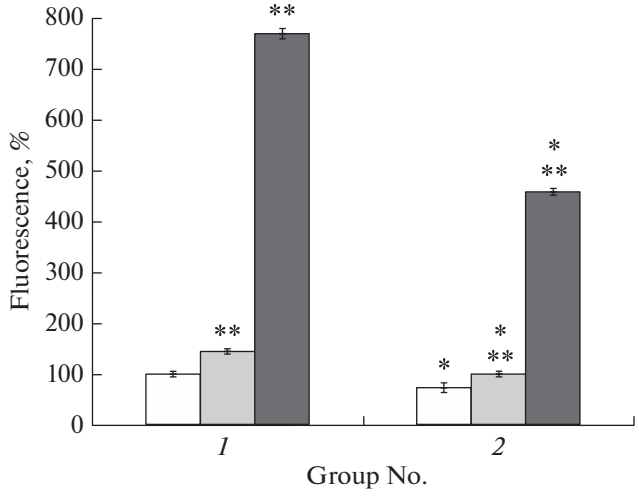

Fig. 6. The effect of a hypomagnetic field on dichlorofluorescein fluorescence in neutrophil suspensions with and without the presence of apocynin. Y-axis: maximum fluorescence intensity as percentage of the basal control (mean values and standard deviations, $n = 10$). X-axis: *1*, control group; *2*, experiment. Open bars: no additives; gray bars: 20 μM apocynin; dark bars: 500 μM apocynin. * Differences between groups significant at $p < 0.05$. ** Differences within a group significant at $p < 0.05$.

with regulatory calcium-dependent mechanisms [9] that control the respiratory burst in neutrophils.

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